THE INFLUENCE OF TSETSE MIDGUT OXIDANTS ON TRYPANOSOME ESTABLISHMENT

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Declaration

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

The research work was undertaken at the Liverpool School of Tropical Medicine (University of Liverpool). DNA sequencing was carried out at the DNA Sequencing core (Cardiff University, Cardiff). The *in vitro* cytotoxicity experiments were performed in the laboratory of Professor Terry Pearson (Department of Biochemistry and Microbiology at the University of Victoria, Canada) by Lee Haines. The tsetse midgut 454 cDNA was prepared by Stella Lehane and was sequenced and assembled by Alistair Darby in association with Professor Neil Hall at the Genomics Suite (The Centre for Genomic Research, University of Liverpool). The unpublished figure 5.3 (top 184 using tagwise dispersions) was obtained from Alistair Darby and has been adapted for this thesis.

Abstract

Tsetse flies (Diptera: Glossinidae) are the vectors of African trypanosomes which cause human sleeping sickness and nagana to animals. Despite their importance as vectors, tsetse flies are largely refractory to trypanosome infections after the first two bloodmeals. Fly immunity is thought to play a crucial role in determining the outcome of initial parasite establishment in the fly midgut. As a result of defending against trypanosomes and haem released during blood digestion, redox genes probably play a significant regulatory role in midgut physiology of tsetse flies. The primary objective of this PhD thesis was to test the hypothesis that superoxides are a core protective device for tsetse flies against invading trypanosomes.

To first examine whether inducible nitric oxide synthase (NOS), a putative pro-oxidant enzyme, acts as one of the barriers to trypanosome establishment in the tsetse midgut, temporary depletion of NOS from the midgut using RNA interference (RNAi) was performed. This caused a significant increase ($p=0.011$) in midgut infection prevalence (2-fold) compared to when NOS was reduced post-infection. It had the most noticeable effect when knockdown occurred before the trypanosome infection event. Further evidence for this was given by the inhibition of NOS by oral administration of the NOS inhibitor L-NAME which also caused a significant increase in midgut infections ($p=0.048$). Further, indirect evidence of the anti-trypanosomal role of NOS is seen in the fact that NOS activity increased almost 2-fold in trypanosome-infected flies compared to uninfected flies. By contrast, in tsetse NOS levels did not change in response to bacterial infections with Gram-negative *Escherichia coli* or Gram-positive *Staphylococcus aureus* suggesting this is quite a specific response to trypanosome infection.

An indirect approach was taken to measure reactive oxygen species (ROS) levels in tsetse flies. ROS levels were correlated with trypanosome prevalence in infected flies under various physiological conditions known to impact susceptibility: fly age, starvation, fly sex, bloodmeal fractions, immune-stimulation with bacteria, knockdown of immune genes (tsetseEP protein) and puparial conditions. The indirect evidence gathered suggested that changes in ROS levels cannot explain changes in susceptibility seen in tsetse under this range of physiological conditions. The only result suggesting a role for ROS in trypanosome infection was the observation of a significant ($p=0.029$) increase in hydrogen peroxide levels in flies infected with live trypanosomes compared to uninfected flies. Consequently, a direct approach was attempted to look for a role of ROS in trypanosome establishment in tsetse midgut. Gene knockdown via RNAi of genes involved in ROS generation (dual oxidase – Duox) and removal (oxidation resistance 1 - OXR1) was attempted. Knockdown of transcript levels of Duox resulted in increased midgut infection prevalence suggesting its involvement in controlling parasite establishment. However, despite repeated attempts, transcript levels of OXR1 could not be knocked down.

A bioinformatic approach including phylogenetic analyses of NOS, Duox and OXR1 was taken for comparative analysis with other dipterans. In addition, manual extension of the sequences of these genes was carried out and the extended protein sequences of these genes are now comparable in length to *Drosophila* orthologs, incorporating necessary domains and residues essential for their functionality. Available resources such as a 454 cDNA tsetse midgut transcriptome enabled the identification of 70 genes which were differentially expressed in tsetse fed trypanosomes. Trypanosome infection was found to have a substantial effect on metabolic and cellular processes in the fly, in addition to the emerging common classes such as serine proteases, adhesion proteins, reactive intermediates and immune-related genes.

Finally, with the recent release of the *Glossina morsitans morsitans* genome, a selection of antioxidant genes was manually annotated to include transcript and protein sequence predictions. Consequently, a comparative analysis with the model system, *D. melanogaster* was carried out. The essential components of the antioxidant system appear to be conserved, with a few exceptions. In addition, lack of differential expansion of antioxidant gene families among *Glossina* and other dipterans including *Drosophila* reflects their close relation. Furthermore, we are now able to validate antioxidant genes identified in the past and find novel genes such as a second thioredoxin reductase in tsetse. Overall, this research involves both functional and bioinformatic approaches to offer new insights into the roles of oxidants in response to trypanosomes in the tsetse fly.

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List of Abbreviations

aa	amino acid
AAT	African animal trypanosomiasis
AMP	antimicrobial peptide
BLAST	basic local alignment search tool
BMAP	bovine myeloid antimicrobial peptide
bp	base pair
bNOS	bacterial nitric oxide synthase
BSA	bovine serum albumin
BSF	bloodstream form
CaM	calmodulin
Cat	catalase
CDD	conserved domain database
ddNTP	dideoxy nucleoside triphosphate
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
Duox	dual oxidase
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
EGTA	ethyleneglycotetraacetic acid
eNOS	endothelial nitric oxide synthase
EP	glu-pro
EST	expressed sequence tag
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GAPDH	glyceraldehyde phosphate dehydrogenase
GFP	green fluorescent protein
GlcN	D-glucosamine
GlcNAc	*N*-acetyl-D-glucosamine
GNBP	Gram-negative binding protein
GPEET	gly-pro-glu-glu-thr
GPI	glycosyl phosphotidyl inositol
GPx	glutathione peroxidase
HAT	human African trypanosomiasis
HDP	host defence peptide
HEPES	hydroxyethyl piperazineethanesulfonic acid
HPX2	heme peroxidise
HSP	heat shock protein
IMD	immunodeficiency
IMPer	immunomodulatory peroxidise
iNOS	inducible nitric oxide synthase
IPTG	isopropyl β-D-1 thiogalactopyranoside
IRC	immune-regulated catalase
Jafrac	thioredoxin peroxidase
kDa	kiloDalton
LB	Luria-Bertani
L-NAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride
L-NMMA	N^{G}-Methyl-L-arginine acetate

LPS	lipopolysaccharide
LSTM	Liverpool School of Tropical Medicine
MEM	minimal essential medium
MIC	minimal inhibitory concentration
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NOX5	NADPH oxidase 5
OD	optical density
ORF	open reading frame
OXR1	oxidation resistance 1
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCF	procyclic culture form
PCR	polymerase chain reaction
p.e	post emergence
PGRP	peptidoglycan recognition protein
PHD	peroxidase domain
PLA_2	phospholipase A2
PM	peritrophic matrix
PPO	prophenoloxidase
PRR	peptidoglycan recognition receptor
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse-transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S.E.	standard error
Serpin	serine protease inhibitor
SFM	serum free media
siRNA	short interfering ribonucleic acid
SNAP	*S*-nitroso-*N*-acetylpenicillamine
SNP	sodium nitroprusside dehydrate
SOD	superoxide dismutase
SRA	serum resistance associated
STE	sodium chloride-Tris-EDTA
TAE	tris-acetate EDTA
TLR	toll-like receptor
TNFα	tumour necrosis factor α
Trxr	thioredoxin reductase
uNOS	universal nitric oxide synthase
VSG	variant surface glycoprotein
WGS	whole genome shotgun sequencing
XGAL	bromo-chloro-indolyl-galactopyranoside
^3H	tritium-labelled

CHAPTER 1

Literature Review

1.1 African Trypanosomiasis

African trypanosomes are protozoan parasites that cause human African trypanosomiasis (HAT) or sleeping sickness, a fatal disease in humans and Animal African trypanosomiasis (AAT) or nagana in animals. HAT places a heavy burden on human health with 17,500 new cases reported per year and an estimated cumulative rate of 50,000 to 70,000 cases (W.H.O. 2010). In recent years, an epidemic of sleeping sickness spread through Democratic Republic of Congo, Angola, Sudan and Uganda affecting several hundred thousand people (W.H.O. 2010). Following large scale intervention the number of new reported cases fell below 10,000 in 2009 for the first time in 50 years, with the estimated number of actual cases at 30,000 (Lindh and Lehane 2011) (Figure 1.1). The number of reported new cases has continued to decrease and in 2010, 7,139 cases were reported (Lindh and Lehane 2011).

HAT is caused by two separate parasites, both of which cause fatal disease if untreated; *Trypanosoma brucei gambiense* causes a chronic disease which takes a few years to reach the advanced stage and it occurs in Central and West Africa. In East and Southern Africa, *T. b. rhodesiense* causes an acute disease which can be fatal within weeks (Barrett 1999). With no vaccine available or in prospect because of antigenic variation of surface proteins, most control efforts have been aimed at diagnosis and treatment of patients (Aksoy 2003). Unfortunately, drugs for treating sleeping sickness have harsh side effects, are toxic and drug resistance is on the rise (Legros *et al.* 2002; Delespaux and de Koning 2007).

Trypanosomiasis also causes over 3 million cattle deaths with annual losses amounting to more than US $4.5 billion (Budd 1999). Trypanosomes impede the economic sector of the African continent by restricting agricultural development and indirectly affecting human health and nutrition by limiting the availability of food across sub-Saharan Africa (Grady *et al.* 2011). The causative agents of AAT include *T. b. brucei*, *T. vivax* and *T. congolense* (Aksoy 2003). Currently, nagana is managed by chemotherapy, chemoprophylaxis and trypanocidal drugs; however drug resistance (diminazene aceturate) in *T. congolense* has been reported (Miruk *et al.* 2008; Chitanga *et al.* 2011).

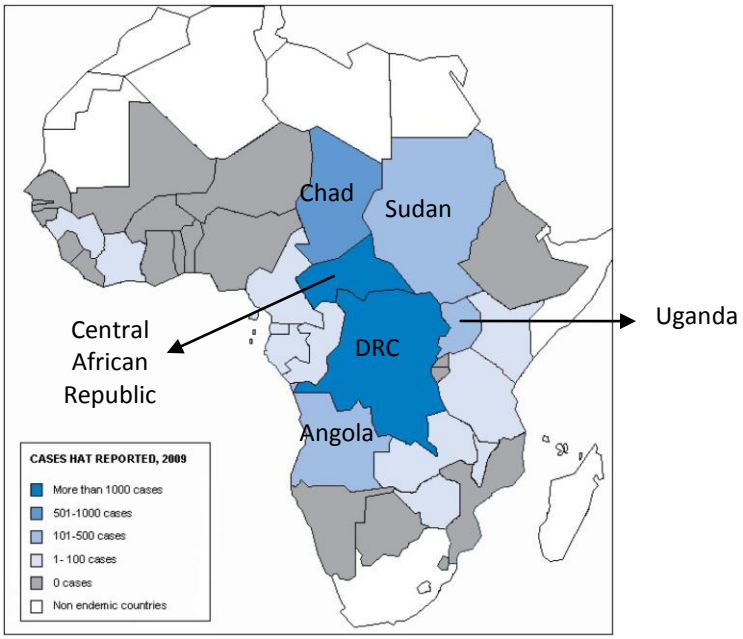


Figure 1.1. Classification of human African trypanosomiasis-endemic countries according to cases reported in 2009. Labelled countries are regions of recently high reported cases. Taken from: (Simarro *et al.* 2011).

Due to the lack of a mammalian vaccine and effective and affordable drugs, control of nagana has been hindered and management of trypanosomiasis now relies heavily on control of the vector, the tsetse fly (Diptera: Glossinidae) with efforts aimed at limiting contact between tsetse, humans and domestic animals (Aksoy 2003). Biological control aimed at altering the tsetse habitats has been used in the past to control tsetse flies; this has included clearing bushes or depleting potential food sources, rendering the habitats unsuitable for tsetse flies (Jordan 1986). Currently, use of insecticides such as synthetic pyrethroids are used for ground and aerial spraying, in addition to the direct application of insecticides using a pour-on formulation to livestock (Torr *et al.* 2007; Torr and Vale 2011). Insecticide impregnated targets and traps baited with odour attractants are also commonly deployed to control tsetse populations in the field (Esterhuizen *et al.* 2011; Torr *et al.* 2011). However, these strategies have been difficult to maintain as disease control programs have been decentralized (Aksoy 2003).

An alternative approach involves the application of the sterile insect technique (SIT) to genetically suppress a population. This involves mass rearing and sustained sequential releases of irradiated sterile males over a target area to eventually diminish the reproductive potential of the target population achieving local control or elimination (Vreysen 2001; Aksoy 2003; Wise de Valdez *et al.* 2011). Although this approach has been

successful in eradicating *Glossina austeni* from Unguja Island, Zanzibar, SIT is very costly (Vreysen *et al.* 2000) and is very unlikely to play a large role in control on mainland Africa. Another potential vector control strategy is based on the paratransgenic expression technology. Since transgenic strategies involving egg manipulation in tsetse are difficult due to the vivaparity of tsetse, exploitation of tsetse's symbiotic associations (the primary obligate mutualistic *Wigglesworthia glossinidia* and the facultative secondary symbiont, *Sodalis glossinidius*) to make transgenic symbionts provide an alternative method (Eissa *et al.* 1996; Cheng and Aksoy 1999). Genetic transformation of symbionts to express trypanotoxic molecules could block parasite transmission and provide manipulation of vectorial capacity of tsetse (Aksoy 2003). Recently, infections with the third tsetse symbiont, *Wolbachia* which resides primarily in reproductive tissues has been shown to confer cytoplasmic incompatibility during embryogenesis in *Wolbachia*-free female tsetse mated with *Wolbachia*-infected male tsetse (Alam *et al.* 2011). This approach could spread parasite resistant traits in natural populations, or on the other hand, the release of *Wolbachia* infected males would reduce fecundity of natural females which are uninfected or infected with a different *Wolbachia* strain, thereby enhancing the current SIT applications without the need to irradiate males (Alam *et al.* 2011).

1.2 Tsetse distribution and physiology

Tsetse flies, of the genus *Glossina* consist of twenty-three recognized species and eight sub-species. These are subdivided into three clades, named after the most identified species in each subgenus: Morsitans, Palpalis and Fusca. These groups are commonly classified according to their ecological niches: savannah (Morsitans), riverine (Palpalis) and forest (Fusca). The Palpalis group displays strong anthrophilicity and contains the most important vectors of HAT while the Morsitans and Fusca groups are generally zoophilic. The Morsitans group contains the most important vectors of AAT although both Palpalis and Morsitans groups are vectors of *T. brucei* sspp (Walshe *et al.* 2009). With the exception of *G. brevipalpis,* the Fusca group is not considered to contain important vectors.

Female tsetse flies typically mate when they are only a few days old (48 to 72 hours post eclosion from the pupae). Females generally mate only once and can store sperm for their lifetime. After fertilization and ovulation of an oocyte, the embryo develops within the uterus and hatches into a larva, i.e. the insects are ovoviviparous. The accessory milk glands of the female tsetse fly supplies the developing larva with a 'milk' secretion made of lipids and proteins. The gestation period lasts 16 days for the first larva and 9 days for

other larvae produced by the female tsetse fly. The larva is deposited as a fully developed third instar, which does not feed. It burrows into the ground and pupariates. The puparium develops into an adult fly four weeks later. Adult flies seek their first bloodmeal within a day of eclosion.

Importantly, tsetse rely solely on blood as a nutritional resource and both male and female flies are blood feeders. Both sexes feed approximately every 24 to 48 hours, and so both sexes are capable of transmitting trypanosomes. Tsetse flies are pool feeders and penetration of vertebrate skin by the proboscis results in the formation of a blood pool beneath the epidermis into which tsetse express their saliva containing anti-haemostatic molecules to facilitate feeding. Trypanosomes are transmitted from the tsetse fly to the mammalian host at this stage. The bloodmeal is ingested by pressure differences from muscular contractions around the oesophagus and a cibarial pump (Lehane 2005). The bloodmeal passes through the proboscis and oesophagus and into a junction within the alimentary canal known as the proventriculus (cardia). The bloodmeal may pass directly into the midgut or be moved into the crop (extension of the foregut) for temporary storage before being passed back into the midgut and the rest of the alimentary canal (Moloo and Kutuza 1970). The proventriculus acts as a valve controlling the passing of blood into the midgut. It is also responsible for producing the peritrophic matrix (PM). Figure 1.2 shows a diagrammatic representation of a female tsetse fly.

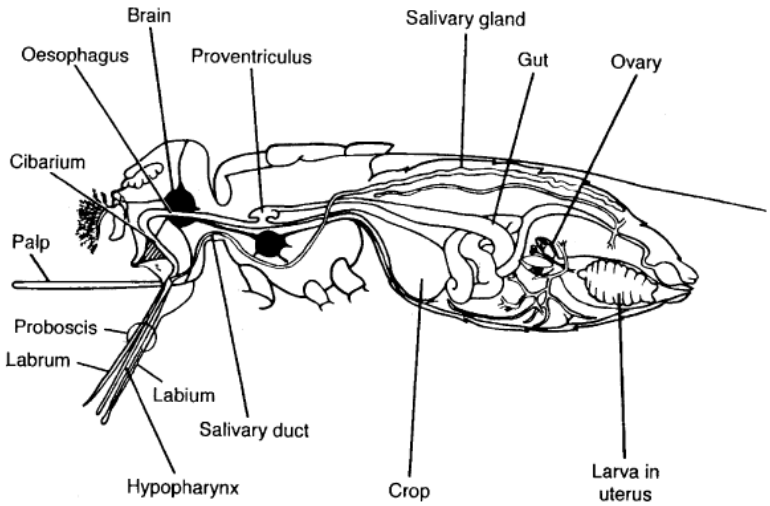


Figure 1.2. Diagram representing a female *Glossina*. The proventriculus at the foregut/midgut juncture, long pair of salivary glands, gut and crop are shown. The ovary and intrauterine larva are also shown. Milk gland ducts which provide the intrauterine larva can be seen surrounding the uterus. Taken from: (Aksoy 2003).

The tsetse midgut which runs from the proventriculus to the Malpighian tubules can be separated into three functional regions: the anterior midgut, the bacteriome and the posterior midgut. The anterior midgut, capable of significant expansion, is responsible for storage of large quantities of ingested blood and rapid dehydration of the bloodmeal (Boehringer-Schweizer 1977). Approximately midway along the anterior midgut, a region of cells known as the bacteriome is located, and this is where the intracellular symbiotic bacteria, *W. glossinidius* reside. Digestion of the bloodmeal and absorption of the digestion products occur in the posterior midgut (Boehringer-Schweizer 1977).

1.3 African trypanosomes

1.3.1 Trypanosome species

Insect transmitted trypanosomes can be categorized into two groups: Stercorarian and Salivarian groups. The Stercorarian trypanosomes are spread via faecal transmission as they develop in the hindgut of insect vectors (tabanids, triatomines, leeches and ticks) (Walshe *et al.* 2009). The salivarian trypanosomes include the causative agents of African trypanosomiasis and are transmitted by the tsetse fly (although some can be transmitted mechanically by tabanids and *Stomoxys* vectors). Salivarian trypanosomes can be classified into the *Nannomonas, Duttonella* and *Trypanozoon* subgenus. The salivarian trypanosomes are transmitted via the mouthparts of tsetse, although the capacity to transmit trypanosomes differs according to tsetse fly species (Leak 1999).

The *Nannomonas* subgenus represents trypanosomes associated with AAT: *T. congolense* (Broden 1904), *T. simiae* (Bruce *et al.* 1912) and *T. godfreyi* (McNamara *et al.* 1994). *T. congolense* is economically important because it can infect a large array of hosts and it is also covers a wide range of geographical distribution. *T. simiae* and *T. godfreyi* are generally associated with infection in pigs. The *Duttonella* subgenus is comprised of *T. vivax* which develops in tsetse proboscis (Haag *et al.* 1998) and has an impact on livestock because of its association with cattle infections. The *Trypanozoon* subgenus is comprised of *T. b. gambiense* and *T. b. rhodesiense* (trypanosomes causing HAT) and *T. b. brucei* (trypanosome causing nagana). A serum-resistance associated (SRA) gene present in *T. b. rhodesiense* enables bloodstream form (BSF) trypanosomes to resist a trypanolytic factor in human serum and cause HAT (De Greef and Hamers 1994; Gadelha *et al.* 2011; Stephens and Hajduk 2011). However, the human-infective *T. b. gambiense* lacks the SRA gene and recently it was demonstrated that reduced expression and mutations to the *T. b. gambiense* haptoglobin-haemoglobin receptor (*TbgHpHbR)* gene confer resistance to

human serum-mediated lysis (Kieft *et al.* 2010). *T. b. brucei* cannot establish infections in humans because of its sensitivity to the trypanolytic factor in human serum (Oli *et al.* 2006; Gadelha *et al.* 2011; Stephens and Hajduk 2011).

1.3.2 *T. brucei* sspp. life cycle

The *T. brucei* sub-species group has the most intricate and perhaps most studied life cycle of all African trypanosome species. The life cycles of *T. brucei* and trypanosomes belonging to the *Nannomonas* subgenus were first described by Robertson in 1913 (Robertson 1913). Since then, a more comprehensive understanding of the developmental stages of trypanosomes has been established. Other trypanosome species have different life cycles including *T. vivax* which occurs in the tsetse proboscis and *T. congolense* which develops in the midgut and matures in the hypopharynx. In addition, the duration of the life cycle of trypanosomes in the fly varies from a few days *(T. vivax)* to a few weeks *(T. brucei)* (Aksoy *et al.* 2003).

Figure 1.3 depicts the life cycle of *T. b. brucei* within the tsetse fly. It starts when a tsetse fly feeds from an infected mammalian host (Hu and Aksoy 2006). In the vertebrate host, two bloodstream form (BSF) trypanosomes are present: long slender and short stumpy. The long slender form replicates by asexual division while the short stumpy form does not replicate. In their mammalian hosts, these extracellular BSF trypanosomes express a thick immunogenic surface coat known as the variant surface glycoprotein (VSG) (Vickerman 1969; Cross 1996). In addition, the VSGs undergo modification by the parasites in a process known as antigenic variation which enables the parasites to evade the host's immune system (Cross 1996). The VSG surface coat guards invariant surface antigens from antibodies, thereby protecting the parasites from the host immune responses (Barry *et al.* 2005). In addition, the presence of a large repertoire of VSG genes allows trypanosomes to switch from expression of one to another creating trypanosome populations with antigenically distinct characteristics within the host (Barry *et al.* 2005; Walshe *et al.* 2009). This strategy allows a minority of trypanosomes to change their expression to another VSG when host immune responses are activated through high parasitaemia, and these parasites can then continue to propagate, causing the next wave of parasitaemia (Barry *et al.* 2005). The continuation of this process leads to chronic infections and allows a lengthened period of parasite transmission (Barry *et al.* 2005).

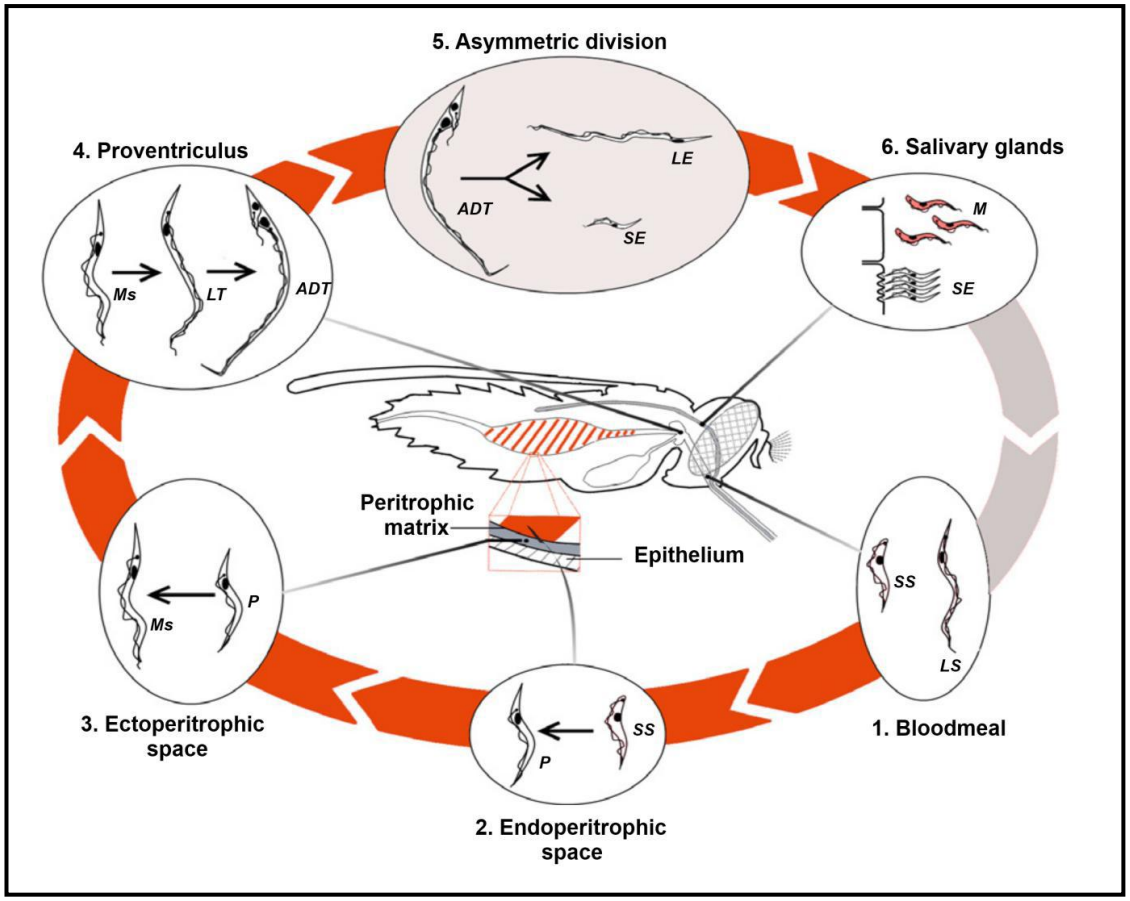


Figure 1.3. Life cycle of *T. b. brucei* within the tsetse fly. The red arrows indicate the trypanosome life cycle within the fly while the grey arrows indicate the transfer between tsetse and its mammalian host. 1: Short stumpy (SS) and long slender (LS) bloodstream forms of trypanosomes are ingested by the fly. 2: Short stumpy forms are transformed into procyclics (P) within the endoperitrophic space of the midgut. 3: Procyclics are transformed into mesocyclics (Ms) within the ectoperitrophic space. 4: Mesocyclics transform into long trypomastigotes (LT) and eventually asymmetrically dividing trypomastigotes (ADT). 5: Asymetrically dividing trypomastigotes (ADT) divide into long epimastigotes (LE) and short epimastigotes (SE). Stage 5 occurs in both the proventriculus and salivary glands. 6: Either the asymetrically dividing trypomastigotes (ADT) or the short epimastigotes (SE) reach the salivary glands, where short epimastigotes (SE) attach to the epithelium of salivary glands and mature into mammalian infective metacyclics (M). Taken from: (Walshe *et al.* 2009).

In the mammalian bloodstream, some long slender BSFs differentiate into short stumpy BSFs, developing a functional mitochondrion, in preparation for the tsetse midgut environment (Vassella *et al.* 1997). Short stumpy BSFs transfer successfully from vertebrate bloodstream (glucose-rich) to the tsetse midgut environment (glucose-limiting) as they efficiently employ glucose and amino acids via the Krebs cycle and oxidative phosphorylation within the mitochondrion (Vassella *et al.* 1997; Aksoy 2003). After transferring into the tsetse midgut, short stumpy BSFs undergo transformation, both metabolic and structural, into procyclic forms within the lumen of the peritrophic matrix in the midgut (Acosta-Serrano *et al.* 2001). In doing so, the surface VSGs are replaced by a new surface coat (procyclin) (Acosta-Serrano *et al.* 2001; Vassella *et al.* 2001); this has been previously demonstrated using *T. b. brucei* trypanosomes expressing green fluorescent protein (GFP) under the control of a procyclin promoter (Gibson and Bailey 2003).

During the first three days, the trypanosomes in the lumen of the anterior midgut move posteriorly and divide exponentially within the bloodmeal as it is being digested. At around three days, an attrition phase begins, leading to the total elimination of infection in a large proportion of flies. In a subset of flies (approximately 10%), the parasites do not get eliminated, and proceed to invade the ectoperitrophic space after 5 days and continue to multiply and move anteriorly (Gibson and Bailey 2003). The reasons for this attrition phase are uncertain but involve tsetse immunity which is discussed later.

The mode of trypanosome migration to the ectoperitrophic space is uncertain although evidence of direct penetration through the peritrophic matrix has been shown (Evans and Ellis 1975; Ellis and Evans 1977; Gibson and Bailey 2003). An alternative suggestion is the migration of trypanosomes around the open, posterior end of the peritrophic matrix although there is no evidence to support this route. From six to eight days post-infection, large numbers of trypanosomes begin to gather in the proventriculus (Van den Abbeele *et al.* 1999; Gibson and Bailey 2003). These trypanosomes stop dividing, and grow into long mesocyclics which later differentiate into long trypomastigotes and undergo asymmetric division into long and short epimastigotes (Van den Abbeele *et al.* 1999). The epimastigotes then migrate into the endoperitrophic space by penetrating through the peritrophic matrix and move anteriorly along the foregut lumen to the tip of the proboscis, entering the opening of the hypopharynx. Suggestions of an alternative route involving direct penetration of the salivary glands via the haemolymph are discounted because of trypanocidal factors in the haemolymph (Mshelbwala 1972; Croft *et al.* 1982). The

asymmetrically dividing trypanosomes or the short epimastigotes then proceed to invade the salivary glands (Sharma *et al.* 2008). The short epimastigotes attach to the epithelium by outgrowths of the flagellar membrane and multiply to colonize the whole salivary gland. Eventually, the epimastigotes differentiate into metacyclics, and this progression is associated with a lesser branched mitrochondrion and Krebs cycle enzymes, migration of the kinetoplast of the parasite to the posterior end, and the appearance of a VSG surface coat. Metacyclics have a small repertoire of VSG genes (Pays *et al.* 2001) and are regulated differently to bloodstream VSGs (Barry *et al.* 1998). Once the parasite detaches from the epithelium of salivary glands and has undergone all the changes, these mature metacyclics are ready to survive within the vertebrate host.

The number of trypanosome infections maturing into transmissible forms relies on various constraints such as fly species (Welburn *et al.* 1989; Welburn *et al.* 1994), fly sex (Distelmans *et al.* 1982; Dale *et al.* 1995), fly starvation (Gingrich *et al.* 1982a) and parasite strain (Dale *et al.* 1995). The salivary glands of an infected tsetse fly remains so for the rest of its entire lifespan (150 days for a female tsetse and about 75 days for a male tsetse fly) (Msangi *et al.* 1998). Therefore, every time a tsetse feeds on a new host, infective metacyclics can be transmitted.

1.3.3 Parasite surface coat

Ingestion of BSF by tsetse results in the cleaving of the parasite's VSG surface coat by a membrane-anchored metalloprotease (MSP B). The VSG is replaced by several million copies of procyclins (Beecroft *et al.* 1993). Procyclins are glycosyl phosphatidylinositol (GPI) anchored proteins and two major forms of procyclins exist: EP and GPEET, which differ in the amino acid repeats in their C-terminus (Acosta-Serrano *et al.* 2001). EP procyclins have glu-pro repeats while GPEET procyclins have gly-pro-glu-glu-thr amino acid repeats (Acosta-Serrano *et al.* 2001). The EP procyclins (EP1, EP2 and EP3) differ in repeat lengths and N-terminal domains and the presence or absence of N-glycosylation sites (Acosta-Serrano *et al.* 2001; Vassella *et al.* 2001). *In vivo,* all four procyclins are present within a few hours of differentiation from BSFs to procyclics (Vassella *et al.* 2001), however at day 3 post-infection, the procyclic coat consists mainly of GPEET (shortened by 11 residues compared to *in vitro* culture procyclic culture forms - PCFs) and reduced amounts of EP forms (Acosta-Serrano *et al.* 2001). At seven days post-infection, midgut procyclins are composed of glycosylated and truncated forms (compared to *in vitro* cultured PCFs) of EP proteins (EP1, EP2 and EP3) (Urwyler *et al.* 2005).

The function of procyclins is unknown. The procyclin N-terminus is susceptible to proteolysis by tsetse midgut proteases while the C-terminal amino acid repeats are resistant to proteolysis (Acosta-Serrano *et al.* 2001). Possible roles of procyclins are protection of parasites from proteases in tsetse, involvement in parasite development and/or ligand-associated parasite signaling within the fly (Roditi and Pearson 1990; Ruepp *et al.* 1997). However, parasites with truncated N-termini procyclins can establish midgut infections and form mature metacyclics within the salivary glands (Liniger *et al.* 2004), suggesting that procyclins are not crucial for migration of procyclics from midgut to salivary glands (Vassella *et al.* 2009). However, procyclin null mutants can establish midgut infections and mature into metacyclics within tsetse salivary glands but are out-competed by wild type trypanosomes in midgut during co-infection experiments, suggesting that procyclins play a role in trypanosome fitness (Vassella *et al.* 2009) although this must be manifested early in the infection process as epimastigotes within the salivary glands lack a procyclin coat, suggesting that the function of procyclins probably occurs in the early stages of trypanosome development in the tsetse fly (Urwyler *et al.* 2005).

1.4 Tsetse-trypanosome interactions

A natural resistance phenomenon known as refractoriness restricts trypanosome transmission in tsetse. Under optimal laboratory conditions where all flies are fed an infected bloodmeal, only a small proportion of flies will establish midgut infections and an even smaller proportion of flies establish midgut infections if they are infected after the third or fourth bloodmeals (Distelmans *et al.* 1982; Kubi *et al.* 2006) (Figure 1.4). In addition, only a small proportion of flies will eventually transmit the parasites to the mammalian host (Hu and Aksoy 2006). Transmission rates vary between 1 and 20% under laboratory conditions (dependent on fly species and parasite strain) (Van den Abbeele *et al.* 1999; Gibson and Bailey 2003), and less than 1 to 5% of the tsetse population is infected with *T. brucei* spp. complex in the field (Woolhouse *et al.* 1993; Lehane *et al.* 2000). The tsetse immune system has been implicated to confer fly refractoriness as flies which were immune-stimulated prior to a trypanosome containing bloodmeal resulted in decreased midgut parasite prevalence (Hao *et al.* 2001).

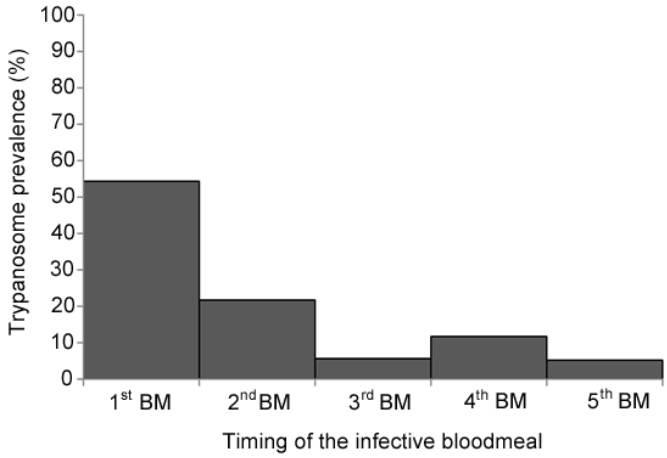


Figure 1.4. Trypanosome prevalence (%) in tsetse *(G. m. morsitans)* midgut and the timing of the infective bloodmeal *(T. b. brucei* TSW196). The infective bloodmeal (BM) fed at one of the stated bloodmeals (x-axis) results in the indicated midgut infection prevalence (y-axis). Data collated from personal results, and from the following: (Hu and Aksoy 2006; Haines *et al.* 2010).

The fruit fly *Drosophila melanogaster* has been used as an effective model system to study innate immunity in insects which is of particular relevance to other dipterans. It is becoming clear from studies in this model organism that innate immunity is a multi-layered host defence system, consisting of physical barriers such as the cuticle and the peritrophic matrix, cellular responses such as phagocytosis and encapsulation, humoral responses such as production of host defense peptides (HDP), formerly called antimicrobial peptides, reactive oxygen species (ROS), nitric oxide (NO) and melanization via the phenoloxidase pathway (Nappi *et al.* 2000; Lemaitre and Hoffmann 2007). In addition, the immune response depends on the pathogen's route of delivery into the tsetse fly to generate either an epithelial or a systemic immune response (Hao *et al.* 2001).

1.4.1 Peritrophic matrix

The peritrophic matrix (previously known as the peritrophic membrane or peritrophic envelope) is a semi-permeable extracellular layer that physically protects the insect midgut epithelium from food abrasion. Tsetse flies have a constitutively expressed type II peritrophic matrix (PM) composed of chitin, a linear polymer of glycosaminoglycans (Tellam *et al.* 1999). In tsetse, the PM separates ingested food from the midgut epithelium by physically separating the midgut lumen into two sections: an endoperitrophic space (where the food is stored) and an ectoperitrophic space (space between the PM and the midgut epithelium) (Lehane *et al.* 1996). The PM is permeable to globular proteins less than 140 kDa as the PM's pore size is approximately 9 nm in tsetse (Miller and Lehane 1990).

The PM acts as a physical barrier against ingested trypanosomes directly invading the tsetse fly, particularly when procyclics need to enter the ectoperitrophic space for development (Figure 1.5). Two migration routes into the ectoperitrophic space have been suggested: parasites enter the ectoperitrophic space at the junction of the PM in the hindgut or directly cross the PM in the midgut (Ellis and Evans 1977). Electron microscopy suggests direct penetration through the PM, and GFP-expressing parasites *T. b. brucei* PCFs have been seen lying parallel to the PM and large numbers of trypanosomes were associated with PM during tsetse midgut dissections (Ellis and Evans 1977; Gibson and Bailey 2003). Although GFP-expressing *T. b. brucei* PCFs were seen in the posterior region of the midgut, they were only present in low numbers and are more likely due to terminal stages of failed infections rather than attempting to enter the ectoperitrophic space via the hindgut (Gibson and Bailey 2003).

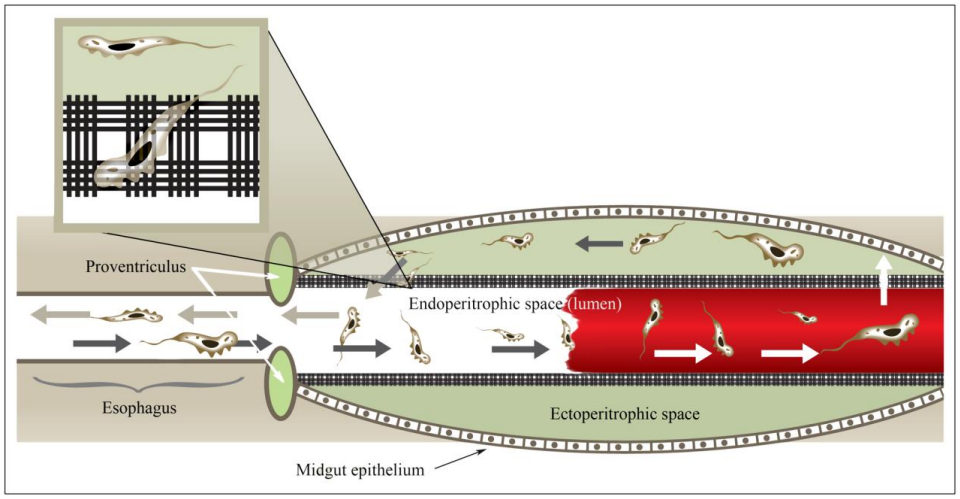


Figure 1.5. Diagram representing the development of African trypanosomes within the tsetse midgut highlighting the double crossing of the peritrophic matrix. (Photo courtesy of Lee Haines).

1.4.2 Immune signaling

Immune events early in the infection process in tsetse appear to affect the disease outcome, suggesting that the fly's immune status at the time of parasite acquisition plays a fundamental role for trypanosome transmission (Hao *et al.* 2001). The first step in an immune response cascade is the recognition of pathogens, followed by stimulation of signaling pathways which leads to the production of immune effector and regulatory molecules (Erturk-Hasdemir *et al.* 2008). Immune signaling pathways are responsible for insect immune responses to a wide range of pathogens. The two major immune signaling

pathways in *Drosophila* which have been extensively studied are the Toll and IMD (immunodeficiency). The Toll pathway, a conserved signaling cascade, is activated upon infections with Gram-positive bacteria and fungi while the IMD pathway is activated upon Gram-negative bacterial infections and is primarily involved in the regulation of epithelial immune responses (Lemaitre and Hoffmann 2007). These two pathways share similar features to the mammalian Toll-like receptor (TLR) and tumour necrosis factor α (TNFα) signaling cascades, and they both regulate NF-κβ transcription factors (Ferrandon *et al.* 2007). Signaling results in the activation and nuclear translocation of NF-κβ transcription factors Dorsal/Diff (Toll pathway) or Relish (IMD pathway) which in turn causes the transcription of host defense peptides (HDPs) (Lemaitre and Hoffmann 2007). Importantly, crosstalk between the two pathways can lead to a wide range of immune responses (Tanji and Ip 2005). The third, less known, pathway is named for the Janus kinases (Jak) and transcription factors (STAT) which control its activation, hence the Jak/STAT pathway. This pathway plays a role against pathogenic bacteria (Buchon *et al.* 2009) and viral infections in *Drosophila* (Dostert *et al.* 2005).

1.4.3 Pathogen recognition

Discriminating between different pathogens is an essential step in eliciting an appropriate immune response. In *Drosophila,* pathogens are recognized by pathogen recognition receptors (PRRs) which interact with pathogen associated molecular patterns (PAMPs). There are two families of PRRs: the peptidoglycan recognition proteins (PGRPs) and the Gram-negative binding proteins (GNBPs). The Toll pathway is activated through pathogen detection of soluble PGRPs which in turn activates the serum protein, Spätzle. The IMD pathway is activated when a pathogen is detected by a membrane-bound class of PGRPs (Paredes *et al.* 2011).

In tsetse flies, the peptidoglycan recognition protein (PGRP-LB) (negative regulator of the IMD pathway in *Drosophila)* has been shown to down regulate the tsetse immune response to potent bacteria (Weiss *et al.* 2008). Adult tsetse flies cured of *Wigglesworthia* have lower PGRP-LB levels than normal flies. PGRP-LB may search for pathogen or symbiont-releasing peptidoglycan to prevent the activation of the host's immune responses which can be damaging to the symbionts (Wang *et al.* 2009). In addition, PGRP-LB may regulate the density of symbionts. Knockdown of PGRP-LB via RNAi led to the activation of the IMD pathway and the synthesis of HDPs subsequently leading to a decrease in the density of the symbiont, *Wigglesworthia* (Wang *et al.* 2009). Furthermore, high levels of PGRP-LB

protects tsetse from trypanosome infections due to the anti-protozoal activity of PGRP-LB. Reduction of PGRP-LB via RNAi led to increased trypanosome infections due to a decrease in the density of *Wigglesworthia,* while infected flies have lower PGRP-LB levels than flies that have eliminated trypanosome infections (Wang *et al.* 2009). When PGRP-LB and IMD pathway functions are interrupted, tsetse flies become more susceptible to trypanosome infections.

1.4.4 Host defence peptides

Host defence peptides (HDPs), also known as antimicrobial peptides (AMPs) are effector molecules produced as part of the humoral defense system. These are small, less than 10 kDa (with the exception of 25 kDa Attacin) cationic molecules and demonstrate a wide range of activities against bacteria and fungi (Lemaitre and Hoffmann 2007). HDPs also play an anti-parasitic role in a number of vectors (Durvasula *et al.* 1997; Shahabuddin *et al.* 1998; Boulanger *et al.* 2002) including the tsetse fly (Hu and Aksoy 2005; Hu and Aksoy 2006). The mode of action of HDPs in targeting pathogens include the disruption of internal cell functioning which can lead to cell death by apoptosis or necrosis and disruption of the pathogen membranes by changing the permeability of the membrane (Walshe *et al.* 2009). HDPs in *G. m. morsitans* include attacin, cecropin, defensin and diptericin. Immune stimulation with live *E. coli* results in an up regulation of attacin and defensin transcripts in the midgut, proventriculus and fat body of tsetse (Hao *et al.* 2001; Hao *et al.* 2003).

When a tsetse fly acquires BSFs by feeding on infected animals, the early stages of the trypanosome infection result in a negligible initial response (Hao *et al.* 2001). However, as the BSFs transform into PCFs and as numbers of parasites increase (by day 6), attacin and defensin expression is high in tsetse fat body (Hao *et al.* 2001; Hao *et al.* 2003). In flies that have eliminated the trypanosome infections (self-cured flies), transcript expression levels of HDPs decrease while transcript levels of HDPs in flies with established midgut infections remain high in the fat body and proventriculus (Hao *et al.* 2001; Hao *et al.* 2003).

In vitro studies with recombinant attacin resulted in the killing of a wide range of pathogens including *E. coli* and the inhibition of both BSF and PCF growth of trypanosomes (Hu and Aksoy 2005; Hu and Aksoy 2006). In addition *in vivo* RNAi knockdown of attacin and relish (an upstream transcriptional activator) resulted in an increase in midgut and salivary gland trypanosome infections (Hu and Aksoy 2006). Refractory species of *Glossina*

showed higher transcript levels of attacin expression in the fat body and proventriculus/midgut than teneral and blood fed susceptible flies (Nayduch and Aksoy 2007). RNAi knockdown of attacin transcript in *G. pallidipes* resulted in higher infection rates than untreated flies although starvation and high mortality rates were observed (Nayduch and Aksoy 2007).

Synthetic AMPs derived from active sites of known AMPs have been shown to be trypanolytic (Harrington 2011). Bovine myeloid antimicrobial peptide-18 (BMAP-18), a truncated form of BMAP-27 is trypanolytic against both BSFs and PCFs trypanosomes and has reduced toxicity to the symbiont *Sodalis* (Haines *et al.* 2009). Synthetic peptides derived from insect defensins have also shown trypanolytic activity against BSFs and PCFs to a lesser degree (Kitani *et al.* 2009; Yamage *et al.* 2009).

1.4.5 Lectins

Lectins are one of the suggested immune factors related to trypanosome susceptibility (Maudlin and Welburn 1987). It was suggested that lectins expressed in tsetse midgut kills trypanosomes in the fly, and that lectin levels are altered by the numbers of *Sodalis* in the fly midgut (Welburn *et al.* 1999). This proposed role of lectins was due to increased midgut trypanosome infection prevalence when flies were fed sugars capable of inhibiting lectins (Maudlin and Welburn 1987; Ingram and Molyneux 1988; Welburn *et al.* 1994). In addition, *in vitro* culture of procyclic forms of trypanosomes with a commercial plant lectin (concanavalin A – Con A) resulted in the killing of trypanosomes and these parasites showed characteristics of apoptotic cells (Welburn *et al.* 1999; Pearson *et al.* 2000). In the tsetse midgut, a lectin specific for D-glucosamine (GlcN) and minor affinity for *N*-acetyl-D-glucosamine (GlcNAc) have been identified, and found to be attached to the PM (Ibrahim *et al.* 1984; Ingram and Molyneux 1988; Lehane and Msangi 1991). The lectin hypothesis is suggested to occur naturally, particularly in newly emerged flies where it has been suggested, but not demonstrated, that lectins are neutralized by the endochitinase of *Sodalis* releasing chitin during pupal development, which in turn produces lectin-inhibitory sugars, suggesting the maternal mode of inheritance of susceptibility to trypanosome infections (Welburn and Maudlin 1999). Although the lectin hypothesis argues that higher densities of symbiont lead to increased susceptibility, the correlation between symbiont densities and susceptibility is absent in some cases calling the hypothesis into question (Weiss *et al.* 2006).

Although inhibition of lectins leads to increased trypanosome survival in tsetse midgut, conversely, lectins are also involved in the maturation of parasites into metacyclic forms (Osir *et al.* 1995; Welburn and Maudlin 1999; Aksoy *et al.* 2003). *In vivo* studies with GlcNAc resulted in trypanosome growth in tsetse midgut, and *in vitro* studies with GlcNAc also promoted growth of PCF trypanosomes (Ebikeme *et al.* 2008). The metabolic impact of GlcNAc on trypanosomes has no relevance to the success of trypanosome establishment within the tsetse midgut, complicating the interpretation of the phenotypes of increased trypanosome prevalence observed (Ebikeme *et al.* 2008). In addition, GlcNAc is an antioxidant molecule which detoxifies free radicals produced by the generation of reactive oxygen species (Xing *et al.* 2006). Detoxification of free radicals provides a more hospitable environment for trypanosomes to survive and establish and this provides a much simpler explanation for the effects of GlcNAc on trypanosome infections in tsetse.

1.4.6 Reactive Oxygen Species (ROS)

The NF-κβ independent production of reactive oxygen species (ROS) is a key component of the insect epithelial immune responses (Lemaitre and Hoffmann 2007). Reactive oxygen species (ROS) are free radicals that are produced from oxidation-reduction (Redox) reactions (Figure 1.6). The free radicals include superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), perhydroxyl radical ($HO_2^{\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl) (Lu *et al.* 2010). In *Drosophila* and mosquitoes, dual oxidase (Duox) a transmembrane protein is responsible for the generation of infection-induced microbicidal ROS (Kumar *et al.* 2003; Ha *et al.* 2005a; Ha *et al.* 2005b; Kumar *et al.* 2010). It has been demonstrated that Duox regulates oxidative bursts in *Drosophila* gut epithelia, generating $O_2^{-\bullet}$ and H_2O_2 onto epithelial surfaces (Ha *et al.* 2005a). In the extracellular space, H_2O_2 can form other radicals such as hydroxyl radical ($^{\bullet}OH$) and hypochlorous acid (HOCl) (Geiszt *et al.* 2003; El Hassani *et al.* 2005) (Figure 1.7). In vertebrates, activated macrophages undergo respiratory bursts in which superoxide anion ($O_2^{-\bullet}$) is produced by NADPH oxidase and transformed to H_2O_2 by superoxide dismutase (SOD), leading to an increase in ROS. Myeloperoxidase then uses H_2O_2 as a substrate to produce hypochlorous acid (HOCl), a highly bactericidal compound.

At low levels, ROS act as signaling molecules (Kuge *et al.* 2001), however at high levels (ROS can be produced swiftly as part of an oxidative burst) ROS can exert oxidative stress leading to damages to DNA, RNA and proteins if surplus ROS are not dealt with. Host cells are generally protected from oxidative damage through the use of intracellular enzymes that

detoxify ROS, thus keeping homeostasis of ROS at a low level. Detoxification enzymes include superoxide dismutase (SOD) that catalyzes the production of H_2O_2 from more reactive peroxide ions ($O_2^{-\bullet}$). Catalase, glutathione peroxidase (GPx) and thioredoxin peroxidase detoxify H_2O_2 into water and non-reactive species.

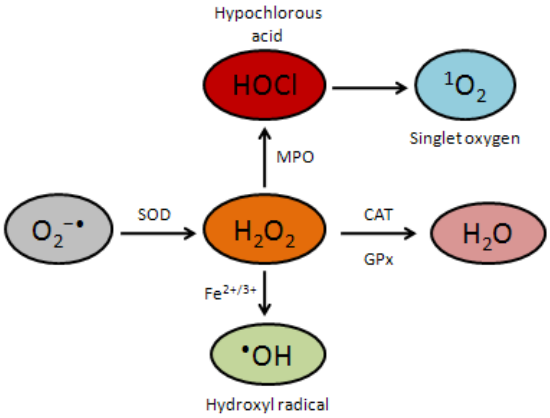


Figure 1.6. Generation and detoxification of reactive oxygen species. Superoxide dismutase (SOD) converts superoxide anion ($O_2^{-\bullet}$) into hydrogen peroxide (H_2O_2), the latter can be reduced by three different pathways. (1) H_2O_2 is reduced into water (H_2O) and oxygen by catalase (CAT) and glutathione peroxidase (GPx). (2) H_2O_2 is converted by myeloperoxidase (MPO) into hypochlorous acid (HOCl). HOCl reacts with H_2O_2 to form singlet oxygen ($^1O_2$) and water. (3) H_2O_2 is catalyzed by the Fenton reaction ($Fe^{2+/3+}$) to form the highly reactive hydroxyl radical ($^\bullet OH$).

Antioxidant molecules are also available to decrease the oxidative damage by either directly reacting with free radicals to eliminate the unpaired condition of the radical or indirectly regulating ROS-related enzymes. These include glutathione, cysteine, *N*-acetyl-cysteine, ascorbic acid and uric acid (Macleod *et al.* 2007b). Two modes of actions have been proposed for the indirect role of antioxidants in decreasing the cellular levels of free radicals. First, antioxidants can inhibit the expression of enzymes that generate free radicals such as NADPH oxidase or xanthine oxidase or secondly, antioxidants can upregulate the expression of antioxidant enzymes such as SOD, catalase and GPx (Lu *et al.* 2010).

1.4.6.1 Roles of ROS in insects

In *Drosophila,* rapid synthesis of ROS is generated upon natural gut infections with bacteria (Lemaitre and Hoffmann 2007; Vallet-Gely *et al.* 2008). ROS are constitutively produced in basal amounts by the N-terminal extracellular peroxidase domain (PHD) of Duox, a member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family (Ha *et al.* 2005a; Ha *et al.* 2005b). However, upon infection, Duox is induced leading to increased production of ROS, with PGRP-LC playing an important role (Ha *et al.* 2005a; Ha *et al.* 2009b). Due to the highly reactive nature of ROS, excess ROS can be detrimental to proteins, DNA and lipids, leading to oxidative stress (Nordberg and Arner 2001). Therefore a fine redox balance of ROS generation and removal is essential in order to prevent the observed increased mortality rates in *Drosophila* lacking the ability to remove ROS (Ha *et al.* 2005a; Ha *et al.* 2005b). A secretory antioxidant enzyme with catalase activity known as immune-regulated catalase (IRC) removes any residual luminal ROS which might harm *Drosophila* gut epithelia (Figure 1.7) (Ha *et al.* 2005b). RNAi knockdown of IRC leads to increased mortality rates due to excessive exerted oxidative stress (Ha *et al.* 2005a; Ha *et al.* 2005b). Recently, an ancient gene present in all eukaryotes, oxidation resistance 1 (OXR1) was identified in *Anopheles gambiae* and was shown to regulate basal levels of the two detoxification enzymes, catalase and glutathione peroxidase (Jaramillo-Gutierrez *et al.* 2010). Systemic injection of H_2O_2 resulted in increased OXR1 transcript levels while OXR1 was found to protect mosquitoes from oxidative stress (Jaramillo-Gutierrez *et al.* 2010).

In dipterans, aging results in increased oxidative stress and loss of efficiency of antioxidant enzymes. Inactivation of catalase in *D. melanogaster* (Mackay and Bewley 1989), *An. gambiae* (Magalhaes *et al.* 2008a), *Musca domestica* (Allen *et al.* 1983) and *Rhodnius prolixus* (Paes *et al.* 2001) all led to increased mortality due to excessive ROS levels. In female *An. gambiae,* fecundity normally decreases with age (DeJong *et al.* 2006). However, this age-related reduction can be reversed with dietary supplementation of antioxidants, which reduce systemic levels of ROS (DeJong *et al.* 2006).

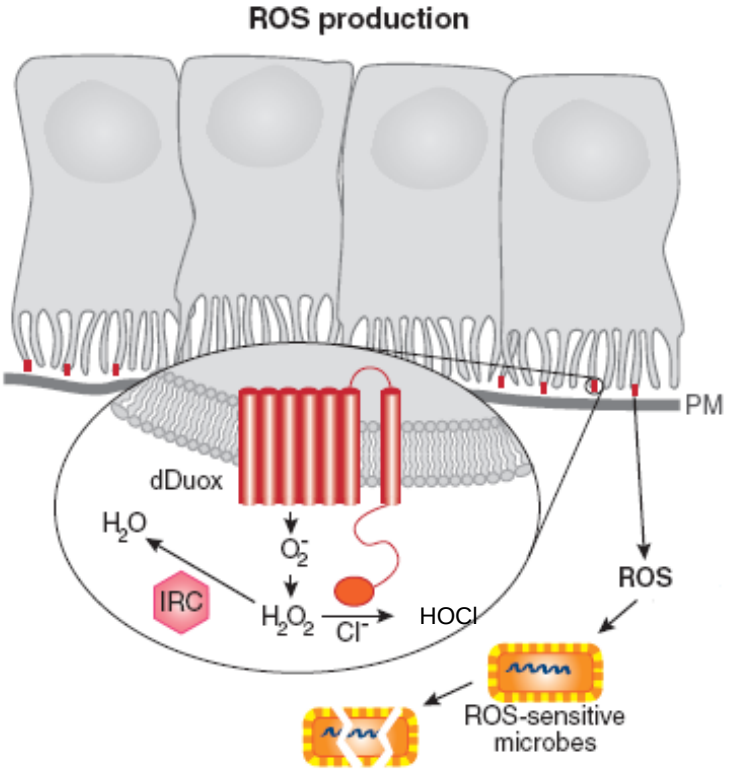


Figure 1.7. Gut immune response of *Drosophila*. The Duox protein produces ROS, and the IRC catalase detoxifies ROS. The PHD domain of *Drosophila* Duox can transform H_2O_2 into HOCl, a highly microbicidal compound. PM, peritrophic matrix. Taken from: (Lemaitre and Hoffmann 2007).

ROS have been implicated to play a role in *An. gambiae* mosquitoes against both bacterial and parasitic infections (Molina-Cruz *et al.* 2008; Cirimotich *et al.* 2011). *An. gambiae* which are refractory to *Plasmodium* infection have higher ROS levels, leading to parasite killing in the midgut, compared to susceptible strains of *An. gambiae* to *Plasmodium* infection (Kumar *et al.* 2003). Administering an antioxidant such as uric acid decreases mortality after *P. berghei* infection, indicating that oxidative stress can be lethal to infected mosquitoes (Molina-Cruz *et al.* 2008). In response to blood feeding, *An. gambiae* mosquitoes secrete a peroxidase from midgut epithelial cells, and the latter together with Duox, catalyze the cross-linking of proteins forming a dityrosine barrier which reduces the permeability to immune elicitors (Kumar *et al.* 2010). This barrier prevents the activation of immune responses against pathogens and creates a friendly environment for *Plasmodium* development (Kumar *et al.* 2010). However, disruption of this barrier results in permeability of immune elicitors, resulting in immune activation and ROS generation (Kumar *et al.* 2010).

ROS has been shown to activate a cell death pathway in *T. b. brucei* procyclics (Ridgley *et al.* 1999; Wang *et al.* 2002). In tsetse, increased H_2O_2 and nitric oxide (NO) levels are induced in the proventriculus following a trypanosome challenge (Hao *et al.* 2003). In addition, genes involved in oxidative stress are induced in midgut transcriptome of infected and self-cured flies (Lehane and Hargrove 1988; Lehane *et al.* 2003; Munks *et al.* 2005). Dietary supplementation of antioxidants such as glutathione, cysteine, *N*-acetyl-cysteine, ascorbic acid and uric acid in the infective bloodmeal leads to considerable increases in midgut trypanosome infections (Macleod *et al.* 2007b). This indirect evidence may suggest that antioxidants detoxify free radicals within the tsetse gut, and thus promote trypanosome survival and establishment. In addition, in the Cape Buffalo, suppressed trypanosome infections is associated with increased levels of ROS in serum (Wang *et al.* 2002).

1.4.7 Nitric oxide synthase (NOS)

In addition to the generation of ROS as part of the innate immune response, reactive nitrogen species (RNS) can also be induced. RNS are free radicals such as nitric oxide, similar to ROS in that they play various essential roles in biological processes but can be harmful due to their reactivity (Fang 2004). Nitric oxide (NO), produced by nitric oxide synthase (NOS), plays an essential role in cellular signaling, vasodilation and immune response (Drew and Leeuwenburgh 2002). In various organisms, the roles of NOS,

specifically inducible NOS and NO are shown to be involved in response to pathogenic infections.

Nitric oxide synthase (NOS) catalyzes the conversion of L-Arginine to L-Citrulline, producing nitric oxide (NO) (Luckhart *et al.* 1998; Andrew and Mayer 1999). NOS consists of two identical monomers, which can be divided into two major domains including a C-terminal reductase domain and an N-terminal oxygenase domain (Andrew and Mayer 1999). The C-terminal reductase domain can bind to NADPH, FAD and FMN, while the N-terminal oxygenase domain binds to haem, BH_4, and the L-arginine substrate (Andrew and Mayer 1999). A calmodulin (CaM) binding domain connects these two regions, and this CaM binding domain plays an important role in both the structure and function of NOS (Andrew and Mayer 1999; Crane *et al.* 2010).

There are three distinct isoforms of NOS, each differing in structure and function, and these include, endothelial NOS (eNOS) and neuronal NOS (nNOS), both constitutively expressed and the third being an inducible NOS (iNOS) (Andrew and Mayer 1999). eNOS and nNOS are Ca^{2+} dependent enzymes although eNOS can be activated in a Ca^{2+} independent approach while iNOS is completely independent of Ca^{2+} increase (Andrew and Mayer 1999).

1.4.7.1 Endothelial NOS (eNOS)

eNOS is a major factor involved in the normal function of the cardiovascular system (Andrew and Mayer 1999). eNOS is constitutively expressed in cells lining blood vessels, and is expressed by cardiac myocytes and cardiac conduction tissue (Andrew and Mayer 1999). eNOS has various specialized functions, and the properties allowing eNOS to perform such particular functions include Ca^{2+} sensitivity and post-translational modifications (Andrew and Mayer 1999). eNOS is activated by an increase in intracellular Ca^{2+}, from either an influx of extracellular Ca^{2+} or release from intracellular stores (Andrew and Mayer 1999). Various factors alter the expression of eNOS, including hypoxia, estrogen and exercise (Andrew and Mayer 1999). In addition, eNOS can be activated by hormones such as catecholamines and vasopressin and platelet-derived mediators such as serotonin and adenosine diphosphate (ADP) (Andrew and Mayer 1999). Activation of eNOS by mechanical forces include shear stress and cyclic strain mediated through G protein activation (Andrew and Mayer 1999). Thus, eNOS produces NO which is responsible for regulating blood pressure (Drew and Leeuwenburgh 2002).

1.4.7.2 Neuronal NOS (nNOS)

nNOS is the largest of the three isoforms with an additional 300 amino acid stretch at the N-terminus (Andrew and Mayer 1999). nNOS differs from the other two isomers in its readiness to catalyze the uncoupled reaction, oxidation of NADPH (Andrew and Mayer 1999). nNOS transfers electrons to haem and oxidises NADPH at a high rate, while eNOS and iNOS are much slower at oxidising NADPH (Andrew and Mayer 1999). Haem plays an important role in dimerisation, and the different isoforms differ with respect to tetrahydropterin (BH_4) and its role in dimerisation. nNOS and eNOS can form dimers without BH_4, while iNOS requires the presence of BH_4 for dimerisation (Andrew and Mayer 1999). nNOS is constitutively expressed in nerve cells, heart and skeletal muscle (Daff 2010). As a result, nNOS functions in regulating the physiological activity of contracting skeletal muscle (Drew and Leeuwenburgh 2002).

1.4.7.3 Inducible NOS (iNOS)

iNOS expression can be induced by inflammatory mediators in most vascular cells, including endothelial cells, cardiac myocytes, smooth muscle cells and macrophages in the mammalian system (Andrew and Mayer 1999; Daff 2010). iNOS produces NO and the latter then acts an essential source of highly reactive molecules generated by its interaction with reactive oxygen intermediates such as superoxide anion and hydrogen peroxide, and by interacting with melanin precursors such as tyrosine and dopamine (Fang 2004). Molecules generated by NO reacting with these oxygen intermediates include peroxynitrite and other reactive nitrogen intermediates, some of which could be potentially deleterious (Fang 2004). These include nitroxyl anion (NO^-), peroxynitrite ($ONOO^-$), nitrosonium ion (NO^+), nitrogen dioxide ($NO_2^\bullet$), nitrous acid (HNO_2) and hydroxyl radical ($^\bullet OH$) (Fang 2004). Some of these reactive nitrogen intermediates have strong antibacterial and antiparasitic properties (Fang 2004). Peroxynitrite acts as a potent leishmanicidal agent *(Leishmania amazonensis) in vitro* (Augusto *et al.* 1996) and is highly toxic to *T. cruzi* (Rubbo *et al.* 1994; Pavanelli and Silva 2010). Thus, in response to inflammatory stimuli, iNOS produces NO which is involved in host defense against pathogens (Drew and Leeuwenburgh 2002).

1.4.7.4 NOS and immunity

Although these three distinct NOS isoforms exist in mammals, it is not yet clear whether all three isoforms of NOS are present in insects; however there is evidence of a single copy of inducible NOS characterized in insects (Luckhart *et al.* 1998; Sudhamsu and Crane 2009;

Crane *et al.* 2010). The roles of iNOS and NO have been investigated in various organisms and it has been found to be involved in response to bacterial infection. For example, decreased iNOS levels led to increased susceptibility of *Drosophila* larvae and adults to both septic and natural infections with Gram-negative bacteria (Carton *et al.* 2009). Nappi *et al.* (2000) also observed that NO played a signalling role in inducing the IMD pathway's effector molecule Diptericin in response to exposure of *Drosophila* larvae to Gram-negative bacteria. The induction of iNOS upon immune stimulation has also been demonstrated in the moth, *Manduca sexta* where challenge with Gram-negative bacteria led to an induction of iNOS in gut tissue (Eleftherianos *et al.* 2009). In addition, in mosquitoes iNOS was induced by bacterial challenge (Hillyer and Estevez-Lao 2010).

There is strong evidence to suggest that iNOS and NO are involved in response to parasitic infections in a range of species. Nitric oxide has also been shown to inhibit growth of *T. congolense* in mice and it has been suggested that NO might be cytotoxic in inhibiting multiplication of the parasites (Lu *et al.* 2011). In mouse macrophages NO acts as a potent molecule against intracellular *L. major* parasites causing cell death of parasites (Zangger *et al.* 2002; Nahrevanian *et al.* 2009). In the triatomine bug, *R. prolixus,* NO regulates *T. rangeli* infections and has also been found to interfere with the *T. cruzi* life cycle *in vivo* and *in vitro* (Vespa *et al.* 1994; Whitten *et al.* 2001). iNOS also indirectly controls the intensity and rate of *Plasmodium* infections in several species of mosquitoes, *An. stephensi, An. gambiae* and *An. pseudopunctipennis* (Dimopoulos *et al.* 1998; Luckhart *et al.* 1998; Herrera-Ortiz *et al.* 2004). NO also activates a systemic immune response in *An. albimanus* upon infection with *P. berghei* (Herrera-Ortiz *et al.* 2011). Recently, it was demonstrated that a haem peroxidase (HPX2) and NADPH oxidase 5 (NOX5) enhance the toxicity of NO in response to mounting an antiplasmodial response (Oliveira *et al.* 2012).

Hao *et al.* explored the possible role of iNOS in tsetse trypanosome interactions by using dietary supplementation with L-NAME (Hao *et al.* 2003). They reported no difference in parasite prevalence in tsetse and concluded that iNOS and NO did not play a significant role in trypanosome midgut establishment (Hao *et al.* 2003).

It has been suggested that NOS, through its effector molecule NO, may act on parasites in one of two ways. Either by being involved directly in the killing of the parasite (Dimopoulos *et al.* 2001) or by NO induction of the IMD signalling cascade (Nappi *et al.* 2000). Whether NO in tsetse acts directly on trypanosomes or has its action as part of a signaling cascade remains to be resolved.

1.5 Factors influencing trypanosome susceptibility in tsetse

A range of factors, outlined below, are known to affect the susceptibility of tsetse flies to trypanosomes.

There is evidence that the sex of the fly influences susceptibility to trypanosome infections *(T. congolense* and *T. brucei)*. The majority of laboratory studies (Burtt 1946; Distelmans *et al.* 1982; Mwangelwa *et al.* 1987; Moloo 1993; Dale *et al.* 1995) suggest that male flies are more susceptible to trypanosome infections, while other fly/parasite combinations have given different results (Moloo *et al.* 1992; Welburn and Maudlin 1992).

The post eclosion age of the flies predicts the prevalence of midgut trypanosome infections (Walshe *et al.* 2011). Under laboratory conditions, newly emerged flies (24 hours post-emergence) are more susceptible to trypanosome infections at the first bloodmeal while older flies (48 hours post-emergence) are more resistant to trypanosome infections (Distelmans *et al.* 1982; Welburn and Maudlin 1992; Kubi *et al.* 2006; Walshe *et al.* 2011). Laboratory-based studies have shown that starvation periods (3-4 days for a teneral fly or 7 days for adult flies) lead to increased susceptibility to *T. b. brucei* or *T. congolense* infection, resulting in higher maturation rates of salivary gland infections (Kubi *et al.* 2006). Thus it is likely in the field that high infection rates observed in older flies (Woolhouse *et al.* 1993; Msangi *et al.* 1998; Lehane *et al.* 2000) compared to laboratory flies is due to starvation events in the wild (Walshe *et al.* 2009). Starvation probably affects susceptibility because of the pressure it places on the high energy costs of mounting an immune response (Schmid-Hempel 2005; Walshe *et al.* 2009).

TsetseEP protein, named for the extensive C-terminal region of glutamic acid-proline repeats, shows high similarity to the repeat region of the EP form of procyclin surface coat of *T. b. brucei* (Haines *et al.* 2010). TsetseEP protein is induced upon injection of live *E. coli* and protects tsetse midguts from trypanosome infections in both *G. m. morsitans* and *G. palpalis palpalis* (Haines *et al.* 2005; Haines *et al.* 2010). *In vivo* reduction of tsetseEP protein by RNAi results in an increase in midgut parasite infection in older flies, thus increasing fly susceptibility (Haines *et al.* 2010).

Puparial incubation temperature influences susceptibility to trypanosome infections. Puparia of *G. m. morsitans* exposed to a period of elevated temperature produce flies which are more susceptible to parasites and develop mature infections (Burtt 1946). Prior immune-stimulus of tsetse flies results in lower susceptibility to midgut infections with *T. b. brucei* (Hao *et al.,* 2001). Removal of serum from the bloodmeal also leads to increased

midgut infection rates (Maudlin *et al.* 1984). PCFs are highly susceptible to vertebrate host serum and this susceptibility has been linked to serum complement activity, where the complement cascade is part of innate host immunity to help fight off infection (Ferrante 1983; Black *et al.* 1999; Ooi 2011).

1.6 Physiology of bloodmeal digestion and its importance to ROS-based defence systems

In a single meal, hematophagous arthropods ingest a volume of vertebrate blood that is several times their unfed body weight (Lehane 1991; Braz *et al.* 2001). This poses a physiological challenge for digestion because of the biochemical nature of the vertebrate blood. When the major blood component, haemoglobin, is digested inside the insect digestive tract, large amounts of haem are released together with peptides and amino acids (Paes *et al.* 2001; Oliveira *et al.* 2007; Oliveira *et al.* 2011). When haem is not bound to proteins, it has possible cytotoxic and pro-oxidant effects (Oliveira *et al.* 2011; Tripodi *et al.* 2011). The pro-oxidant activity of haem acts as a catalyst to form potentially toxic radicals, converting lipid hydroperoxides of low reactivity (ROOH) into highly reactive peroxyl ($ROO^{-\bullet}$) and alcoxyl ($RO^{-\bullet}$) radicals with high cytotoxicity (Oliveira *et al.* 2011). This can result in damage to biomolecules such as proteins, carbohydrates and lipids (Oliveira *et al.* 2007). Moreover, haem can associate with phospholipid membranes by unsettling their physical integrity (increasing their permeability) and leading to cell disruption (Schmitt *et al.* 1993). Therefore, the blood feeding insect must find means of coping with the pro-oxidant effects of ingested haem because the combined effect of bloodmeal-derived haem and ROS generated from Duox upon immune-stimulation could be detrimental to the insect (Ha *et al.* 2005a; Kumar *et al.* 2010; Oliveira *et al.* 2011).

Efficient coping mechanisms are in place to protect against the toxic effects of haem and ROS in different hematophagous organisms (Oliveira *et al.* 1999). One of the detoxification mechanisms includes haem degradation through haem oxygenase by a multistep process utilized by the kissing bug, *R. prolixus* (Lara *et al.* 2005; Paiva-Silva *et al.* 2006). Another detoxification mechanism, haem aggregation, involves the sequestration of haem into an insoluble dark-brown pigment called haemozoin, which is less reactive and can subsequently be eliminated in insects' faeces (Oliveira *et al.* 1999; Oliveira *et al.* 2007). In the gut lumen of *R. prolixus,* enzyme systems based on perimicrovillar membranes (phospholipid bilayer membranes covering epithelial midgut cells) convert haem into haemozoin (Oliveira *et al.* 1999; Oliveira *et al.* 2000b; Silva *et al.* 2007). Haemozoin production has been shown in the helminth *Schistosoma mansoni* (Oliveira *et al.* 2000a),

the trematode *Echinostoma trivolis* (Pisciotta *et al.* 2005), the malaria parasite, *Plasmodium* (Slater *et al.* 1991; Slater and Cerami 1992), and the avian protozoan *Haemoproteus columbae* (Chen *et al.* 2001).

Another mode of detoxification involves the expression of antioxidant enzymes such as catalase (Paes *et al.* 2001; Jaramillo-Gutierrez *et al.* 2010; Diaz-Albiter *et al.* 2011) and through haem-binding proteins (Oliveira *et al.* 1995; Paiva-Silva *et al.* 2002). Haem-binding proteins such as HeLp, haem-lipoprotein from the cattle tick *Boophilus microplus* (Maya-Monteiro *et al.* 2004) and RHBP, haem-binding protein from *R. prolixus* (Oliveira *et al.* 1995) can also bind to haem and act as antioxidant proteins (Silva *et al.* 2007). Iron-binding proteins such as transferrins and ferritins have been putatively linked to protection against cytotoxic effects associated with tsetse's hematophagous lifestyle, especially since both sexes of tsetse are blood feeders (Strickler-Dinglasan *et al.* 2006). In addition, higher expression of antioxidant genes (catalase, superoxide dismutase and peroxiredoxin) have been observed in the posterior midgut of tsetse, suggesting a possible mechanism to cope with haem associated oxidative stress from the bloodmeal (Munks *et al.* 2005).

1.7 Study aims

The primary objective of this work was to test the hypothesis that superoxides are a core protective device for tsetse flies against invading trypanosomes. Tsetse flies are believed to generate superoxides as part of the immune response against trypanosomes, thereby increasing the impending oxidative stress in the tsetse midgut (Hao *et al.* 2003). In addition, the obligate blood feeding nature of tsetse flies creates a physiological challenge to the fly. As a result of defending against trypanosomes and blood digestion, antioxidant genes probably play a significant regulatory role in midgut physiology of tsetse flies (Munks *et al.* 2005).

In this current study, functional analysis of the roles of some of the previously identified tsetse antioxidant genes was carried out (Lehane *et al.* 2003; Munks *et al.* 2005; Attardo *et al.* 2006), along with newly identified antioxidant genes (Jaramillo-Gutierrez *et al.* 2010). This study first examines whether inducible NOS acts as one of the barriers to trypanosome establishment in the tsetse midgut (Chapter 2). An *in vivo* reverse genetics approach was used to study the effects of gene knockdown of NOS by RNAi as well as manipulating exogenous levels of NOS. An indirect approach was then taken to measure ROS levels in tsetse flies exposed to various physiological conditions known to impact susceptibility in

flies (Chapter 3). Genes involved in ROS generation and removal, (Duox and OXR1 respectively) were then knocked down by RNAi and the subsequent phenotype of midgut trypanosome prevalence was examined (Chapter 4).

A bioinformatic approach including phylogenetic analyses was used for a comparative analysis of the antioxidant genes with other dipterans. With the available resources such as a 454 platform, attempts were made to compare cDNA sequencing and differential gene expression between uninfected and infected/self-cured flies as well as manually extending the sequences of NOS, Duox and OXR1 in order to incorporate necessary domains and residues obligatory for their functionality (Chapter 5). Finally, with the recent release of the *Glossina* genome, a selection of antioxidant genes was manually annotated, predicting transcript and protein sequences, and a comparative analysis with *D. melanogaster* was subsequently carried out (Chapter 6).

CHAPTER 2

The effect of inducible nitric oxide synthase (NOS) on pathogenic infections in the tsetse fly

2.1 Introduction

Most mature tsetse flies are refractory to trypanosome infections, however the mechanisms behind the process of self-curing are not understood (Krafsur 2009; Walshe *et al.* 2011). Activation of the insect immune processes, such as the production of antimicrobial peptides and other effector molecules, are thought to influence vector competence (Luckhart *et al.* 1998; Hu and Aksoy 2006; Carton *et al.* 2009; Harrington 2011). Our attention has been drawn to one of these effector molecules called nitric oxide synthase (NOS). NOS is an enzyme that catalyzes the conversion of L-Arginine to L-Citrulline, and in the process produces a free radical nitric oxide (NO) (Figure 2.1), which is an important component of innate immunity in the midgut lumen of mosquitoes (Peterson *et al.* 2007; Gupta *et al.* 2009; Ali *et al.* 2011; Vijay *et al.* 2011).

Figure 2.1. The NOS catalyzed reaction. NOS, an enzyme that catalyzes the conversion of L-Arginine to L-Citrulline and produces nitric oxide.

NOS produces NO, which acts as an essential source of highly reactive molecules that interact with reactive oxygen intermediates (Fang 2004). Some of the molecules generated by NO and these oxygen intermediates have strong anti-bacterial and anti-parasitic properties, and are also involved with mammalian cellular immunity (Fang 2004). *Anopheles stephensi* possesses a single copy NOS gene that is inducible upon *Plasmodium* infections (Luckhart *et al.* 1998). In vertebrates, there are three distinct NOS isoforms on separate chromosomes: two constitutively expressed forms, endothelial NOS (eNOS) and neuronal NOS (nNOS) and the third being an inducible NOS (iNOS) (Luckhart and Li 2001). The latter isoform is highly conserved and is the only isoform of NOS characterized in insects (Andrew and Mayer 1999).

It has been previously shown that NOS plays a central role in controlling both the intensity and rate of *Plasmodium* infections in *Anopheles* midguts (Dimopoulos *et al.* 1998; Luckhart *et al.* 1998). NOS and NO have also been suggested to play important roles in early stages of *Drosophila's* immune response to parasitic infections (Carton *et al.* 2009). Hao *et al.* (2003) investigated the possible role of NOS in tsetse, and concluded that NOS and NO were not involved in trypanosome establishment in the fly midgut based on observations in

midgut parasite prevalence between control flies and flies supplemented with NOS inhibitor and NOS substrate.

This study sets out to re-examine the apparent contradictions between the effects of NO in mosquitoes (Dimopoulos *et al.* 1998; Luckhart *et al.* 1998; Herrera-Ortiz *et al.* 2004; Herrera-Ortiz *et al.* 2011) and tsetse (Hao *et al.* 2003). We examine whether inducible NOS acts as one of the barriers to trypanosome establishment in the tsetse midgut. This chapter utilizes two means of manipulating the levels of NO in tsetse flies. Firstly the use of RNA interference (RNAi) was utilized as a technique that causes specific, post-translational gene knockdown by introducing sequence specific double stranded RNAs (dsRNAs) that degrade mRNA (Fire *et al.* 1998). Secondly, pharmacological inhibitors and donors of NOS were used to manipulate exogenous levels of NOS and observe their effects. Consequently, our findings strongly imply that NOS and NO are involved in controlling trypanosome establishment in the tsetse fly midgut. This chapter also demonstrates that the pre-existing condition of the tsetse midgut environment is crucial to whether parasites are able to establish, explaining the previous apparent discrepancies between the role of NOS and NO in mosquitoes and tsetse.

2.2 Materials and methods

2.2.1 Fly maintenance

The colony of *Glossina morsitans morsitans* was maintained at the Liverpool School of Tropical Medicine. Flies originated from Zimbabwe, and were established in LSTM in 2002 from the Bristol colony (Professor Wendy Gibson). Temperature in the insectary was kept at 26°C and 70% relative humidity. Flies were fed defibrinated horse blood (TCS Biosciences Ltd., Buckingham, UK) every 48 hours by using an artificial membrane feeding system (Moloo 1971).

2.2.2 Trypanosome stocks

Trypanosoma brucei brucei strain TSW196 bloodstream form (BSF) parasites and *T. congolense* strain 1/148 BSF were used in trypanosome infection experiments. Parasites were obtained from rats at peak parasitaemia and blood stabilates were frozen at -80°C until use. Two hundred µl of infected mouse stabilate was added to five ml of defibrinated horse blood to give a final parasite concentration of ~5 x 10^5 trypanosomes/ml of *T. b. brucei* and 1.7 x 10^7 trypanosomes/ml of *T. congolense*. Unfed flies were removed the following day. Procyclic culture forms (PCF) of *T. b. brucei* TSW196 were transformed from their corresponding BSF (Brun and Schonenberger 1979) and weaned onto SDM:DTM (50/50) as described (Stebeck *et al.* 1995). PCFs were harvested in log-phase growth and the culture was counted using a Neubauer haemocytometer and adjusted to 7 x 10^6 trypanosomes/ml in defibrinated horse blood. PCF culture was adjusted to 2 x 10^4 trypanosomes/ml for cytotoxicity assays.

2.2.3 Insect cell culture

The *An. gambiae* cell line Sua 5.1 derived from neo-natal larvae (Muller *et al.* 1999) (gift from Amy Lynd) were cultured in Schneider's medium (PromoCell, UK) supplemented with 10% fetal bovine serum (InVitrogen, Paisley, UK), 100 U/ml Penicillin and 100 µg/ml Streptomycin (PromoCell, UK). Cells were cultured at 28°C. The *D. melanogaster* S2 cell line derived from 20-24 hrs old embryos (Schneider 1972) (gift from Juliana Alves-Silva) were cultured under the same conditions.

2.2.4 Bacterial infection

Escherichia coli strain K12 RM148 and *Staphylococcus aureus* strain SH1000 were grown in LB broth and incubated at 37°C with shaking until an OD_{600}=0.5 was reached. Heat-killed bacteria were prepared by incubating *E. coli* cells at 75°C for 10 minutes and *S. aureus* cells at 100°C for 15 min. Bacterial death was confirmed by streaking 200 µl of each heat shocked batch onto LB plates and checking for bacterial growth/death after incubation at 37°C 24 and 48 hours after. Bacterial suspension (100 µl) was diluted in 1 ml of defibrinated horse blood and subsequently fed to flies.

2.2.5 RNA extraction from collected tissue

Tsetse flies were dissected from various tissues including midgut, salivary gland, fat body, reproductive tissue (testes), flight muscle and whole fly. Tissues were dissected in PBS, snap frozen in liquid nitrogen and kept at -80°C prior to RNA extraction. Tissues were homogenized in 500 µl TRIzol reagent (Invitrogen, Paisley, UK) in a 1.5 ml tube, using a battery operated pestle. Homogenized samples were incubated for five minutes at room temperature then 100 µl of chloroform was added and tubes were shaken vigorously by hand for 15 seconds and left to incubate at room temperature for three minutes. Samples were centrifuged at 12,000 g for 15 minutes at 4°C (Jouan, France). The upper aqueous phase was transferred to a separate RNase free tube, and 250 µl of isopropanol was added. Samples were left to incubate at room temperature for ten minutes and subsequently centrifuged at 12,000 g for ten minutes at 4°C. The supernatant was then carefully removed and the RNA pellet was washed once in one ml 75% ethanol (made with DEPC treated water). Samples were mixed by vortexing and centrifuged at 7,500 g for five minutes at 4°C. The supernatant was carefully removed and the RNA pellet was dried briefly for five minutes. The RNA pellet was then resuspended in 20 µl nuclease free water. RNA was quantified using a Nanodrop ND-1000 (Wilmington, DE) spectrophotometer. All tissues were diluted to 50 ng for use in a semi-quantitative analysis of transcript expression using the Promega Access RT-PCR System (Promega).

2.2.6 RT-PCR analysis and assessment of transcript abundance

RT-PCR analysis was performed on RNA extracted from tsetse tissues to confirm transcript knockdown of NOS. Primers for NOS RT-PCR reaction included:

NOS Forward 5'- GGCCTGCGAGACGTTTTGTTTAG-3'

NOS Reverse 5'- TCGCCGGGCTCATAGTCCA-3'

The *G. m. morsitans* housekeeping gene, GAPDH (Accession number DQ016434) was used as a loading control, and primers included:

GAPDH Forward 5'-CTCAGCTTCTGTGCGTTG-3'

GAPDH Reverse 5'-AGAGTGCCACCTACGATG-3'

The RT-PCR cycling conditions included: 45°C for 45 minutes, 95°C for two minutes, 30 cycles of 95°C for 45 seconds, 55°C for one minute, 68°C for one minute and a final extension of 68°C for five minutes. RT-PCR products were analyzed by gel electrophoresis using 1.5% agarose gel. Band intensities were measured using Gene Tools software on a Gene Genius Bio Imaging System (Syngene, Cambridge, UK). A 25 µl RT-PCR reaction consisted of 1 µl template RNA, 12.5 µl AccessQuick 2X mastermix (Promega, Madison, WI, USA), 1 µl forward primer, 1 µl reverse primer, 0.4 µl AMV reverse transcriptase (5 U/ µl) (Promega, Madison, WI, USA) and 9.1 µl nuclease-free water.

2.2.7 NOS transcript expression in insect cell lines

To extract DNA from insect cell lines, cells were homogenized in 200 µl STE buffer (0.1 M NaCl, 10 mM Tris HCl at pH 8 and 1 mM EDTA at pH 8). Samples were then heated to 95°C for 10 minutes and centrifuged at full speed for 3 min to pellet the cellular debris. The supernatant was used as the DNA template in a PCR reaction using NOS primers to check for transcript expression. The 25 µl PCR reaction consisted of 1 µl reaction template, 2.5 µl master mix buffer (New England Biolabs, Ipswich, MA, USA), 2.5 µl dNTP (2 mM), 2.5 µl $MgCl_2$ (25 mM), 1 µl of forward primer (25 nM), 1 µl of reverse primer (25 nM), 0.1 µl Taq polymerase (5 U/µl) (New England Biolabs, Ipswich, MA, USA), and 14.4 µl nuclease-free water. PCR cycling conditions included an initial denaturing step at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 2 min and a final extension of 72°C for 1 min. The PCR products were then loaded onto a 1% ethidium bromide stained agarose gel made with TAE buffer, and visualised under UV light. RNA was also extracted from the cell lines as described in section

2.2.2 with the exception of the TRIzol reagent being added to the cells instead of dissected tissues. An RT-PCR was then performed on samples diluted to 60 ng/µl.

2.2.8 Template gene sequences

Blast sequence similarity searches utilized EST data available from GeneDB database (http://www.genedb.org). The putative NOS clone identified from the *G. m. morsitans* salivary gland EST library was labelled as GMsg-7530 (1,839 bp). Six members constructed the clone, these included GMsg01b11.q1k, GMsg41g05.q1k, GMsg03e07.q1k, GMsg41g05.p1k, GMsg21b08.q1k and GMsg44e09.q1k. PCR analysis was performed using RT-PCR primers to ensure correct fragment size of 418 bp. The longest insert (GMsg01b11.q1k) was selected and transformed into *E. coli* XL1 Blue cells (Stratagene, California, USA) using the heat shock transformation method. Briefly, 100 µl of XLI cells was added into pre-cooled tubes together with 1 µl of GMsg01b11.q1k and left to incubate on ice for 20 minutes. The tube was heated in a water bath at 42°C for 1 minute, and subsequently placed on ice to cool for 2 minutes. This was followed by the addition of 400 µl sterile LB medium (4 g NaCl, 4 g tryptone and 2 g yeast extract) and left to incubate for 45 minutes at 37°C with shaking in order to develop ampicillin antibiotic resistance. The solution was then plated onto ampicillin/LB plates. PCR on single cell isolation was performed to determine if the insert is present.

The plasmid containing the NOS insert was extracted using the Miniprep Spin Kit (Qiagen, California, USA). Briefly, the transformed overnight cell culture was pelleted using a Jouan tabletop centrifuge at 1,900 g for 10 minutes. The supernatant was discarded and the pellet was left in the tube. The pellet was re-suspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube. Buffer P2 was added (250 µl) and mixed thoroughly by inversion followed by the addition of buffer N3 (350 µl) and mixed thoroughly by inversion. The solution was centrifuged for 10 minutes at 12,500 g in a table-top microcentrifuge. The resulting supernatant was transferred into a QIAprep spin column. The solution was then centrifuged for 30 to 60 seconds and the flow through was discarded. Buffer PE was added (750 µl) to wash the QIAprep spin column and centrifuged for 30 to 60 seconds. The flow through was discarded, and the remaining solution was centrifuged for one minute. The QIAprep column was then placed in a clean 1.5 ml tube and 50 µl elution buffer was added to DNA and let to stand for one minute and then centrifuged for one minute. Restriction enzyme EcoRI from Promega (Madison, WI, USA) was used for the enzyme digestion. Restriction enzyme buffer (10 X; 2 µl) was added to two µl of bovine serum albumin (one

mg/ml) and one µl of Eco RI was added. The plasmid was then added (one µl) and the volume of the reaction was made up to 20 µl with nuclease free water. The reaction mix was centrifuged briefly at 12,000 g in a microcentrifuge to collect the contents to the bottom of the tube. It was left to incubate for four hours at 37°C. The resulting product was visualized using gel electrophoresis on a 1% agarose gel.

2.2.9 Synthesis of dsRNA

PCR amplicons tailed with T7 promoter sequences were used to synthesize dsRNAs using the MEGAscript High Yield T7 Transcription kit (Ambion, Huntingdon, UK) according to manufacturer's instructions. NOS DNA plasmid (GMsg01b11.q1k) was used as a template to generate dsNOS. Double stranded enhanced green fluorescent protein (eGFP) was used as a negative control with the cloning vector pEGFP-N1 (Clontech; GeneBank Accession number U55762) being used as a template for eGFP dsRNA synthesis. Primer sequences are listed below, with T7 promoter sites underlined.

ds NOS Forward: 5'- TAATACGACTCACTATAGGGTTCGGCCTATCCAAACTTC -3'
ds NOS Reverse: 5'-TAATACGACTCACTATAGGGTCCTCGACACCAGCCAGAC -3'
dseGFP Forward: 5'-TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTTC -3'
dseGFP Reverse: 5'-TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGCC -3'

PCR cycling conditions included: 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and a final extension of 72°C for 1 minute. dsRNA was purified using MEGAclear™ columns (Ambion) and eluted in nuclease free water. dsRNA concentrations were measured using a Nanodrop ND-1000 (Wilmington, DE) spectrophotometer. Eluates were concentrated in a Christ (Osterode, Germany) 2-18 rotational vacuum concentrator to obtain dsRNA concentrations of 3 µg/µl. Figure 2.2 shows the location of the knockdown construct in relation to the NOS protein.

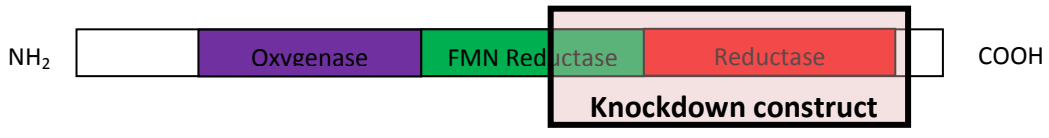


Figure 2.2. Schematic representation of the location of the NOS knockdown construct on the NOS protein. The NOS protein is described with the labelled conserved domains and the location of the knockdown construct is displayed showing that it partially bridges both reductase domains.

2.2.10 Gene knockdown by dsRNA injection

Needles for injections were made using borosilicate glass capillaries (2.00 mm outside diameter) pulled using a needle puller (PC10; Narishige, Japan) to an approximate external tip diameter of 45 µm. Flies were injected 24 hours after receiving a bloodmeal. Flies were chilled on ice and injected with 2 µl of dsRNA into the dorsolateral surface of the thorax (scutum). The needle was angled slightly dorsally to avoid any damage to the organs present in the ventral half of the thorax. Flies were fed 24 hours later or 48 hours thereafter.

Initially, a titration experiment was performed with varying concentrations of dsRNA (1.5 µg, 3 µg, 4.5 µg, 6 µg) in order to determine the optimal dsRNA concentration needed to achieve maximal gene knockdown with minimal mortality. After optimal dsRNA concentration was determined, mortality rates were closely monitored 1 hr, 24 hrs, 48 hrs, 72 hrs and 96 hrs post-injection. Fly tissues (midguts and reproductive tissues) were collected to check for transcript knockdown. RNA was extracted from the tissues, diluted to 50 ng/µl and RT-PCR was conducted using NOS RT-PCR primers. Products were then run on a 1.5% agarose gel.

To check how long knockdown was maintained, a timecourse experiment using tsetse midguts was performed after injecting 6 µg (optimum concentration of dsRNA chosen) of dsNOS and measuring transcript expression from 1, 3, 5, 8, 11 and 13 days post dsNOS treatment. To confirm knockdown, tissues were dissected 7 days post-injection, RNA was extracted and RT-PCR was used to analyze transcript abundance. Band intensities were measured using Gene Tools software on a Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

2.2.11 Gene knockdown and susceptibility to infection

Early knockdown/late infection (2nd bloodmeal) (Figure 2.3 Panel A): All flies were fed a regular bloodmeal on the day of emergence (<24 hours post-emergence) and unfed flies were removed. Fed flies were then injected 24 hours later with 2 µl of 3 µg/µl of dsRNA (NOS or eGFP) or remained uninjected (control). All flies were then fed a trypanosome infected bloodmeal (*T. b. brucei* BSFs or *T. congolense* BSFs) 24 hours after injection, and flies that did not feed were removed. Fly midguts were dissected a week after infection (i.e. adult flies were 10 days old at dissection) and scored for trypanosome infections. Midguts of flies were shredded into 100 µl of saline on a glass slide and infection status was

scored by searching for motile trypanosomes in 10 random fields using dark field microscopy (125 X magnification). Excised midguts (one midgut per tube) were collected into 1.5 ml Eppendorfs in liquid nitrogen, and subsequently stored at -80°C until RT-PCRs were performed on tissues to check for transcript knockdown of NOS.

Early knockdown/ late infection (5th bloodmeal) (Figure 2.3 Panel B): All flies were fed a regular bloodmeal on the day of emergence and unfed flies were removed. Flies were then injected 24 hours later with 2 µl of 3 µg/µl of dsRNA (NOS or eGFP) or remained uninjected (control). Flies were maintained on normal blood until the fifth bloodmeal where flies were fed *T. b. brucei* BSFs infected blood, and unfed flies were removed. Fly midguts were dissected 7 days post-infection (i.e adult flies were 16 days old at dissection) and scored for parasite infection as described above. Midguts were collected to check for transcript knockdown via RT-PCR.

Early infection (1st bloodmeal)/late knockdown (Figure 2.3 Panel C): Late knockdown was accomplished by infecting male flies (<24 hours post-emergence) in the first bloodmeal with *T. b. brucei* BSFs. Unfed flies were removed. Flies were then injected with dsNOS or dseGFP or remained uninjected (control) 24 hours after the infectious bloodmeal. Midguts were dissected 7 days later (i.e. adult flies were 9 days old at dissection) to score parasite infection as described above. Midguts were collected and RT-PCR was carried out to check for knockdown.

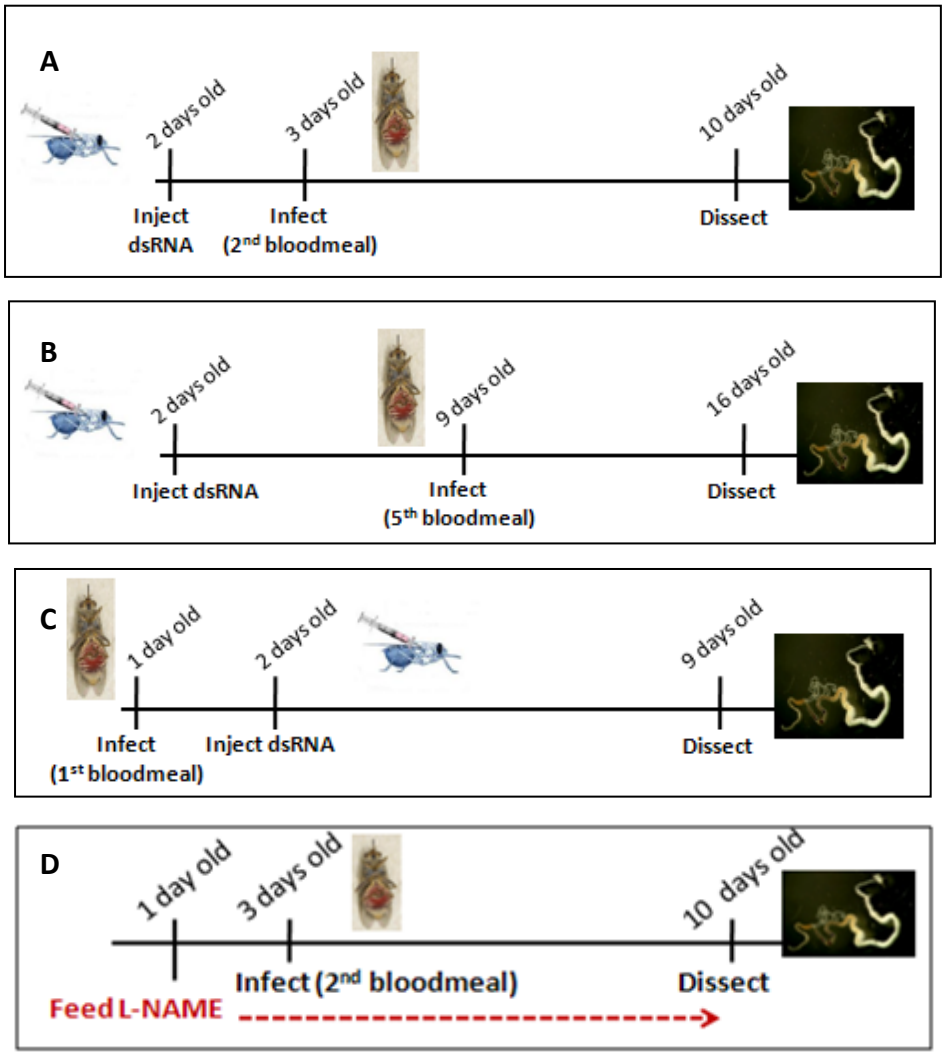


Figure 2.3. Timing of NOS knockdown, infection and dissection and dietary supplementation of NOS inhibitor, L-NAME. (A) Early knockdown followed by infection with *T. b. brucei* and *T. congolense,* and dissection a week later. **(B)** Knockdown at day two, infection with *T. b. brucei* seven days later, dissection a week later. **(C)** Infection with *T. b. brucei,* knockdown a day later and dissection a week later. **(D)** Dietary supplementation of L-NAME, followed by infection with *T. b. brucei* two days later and dissection a week later.

2.2.12 Oral administration of NOS inhibitor/substrate

4 mM of a NOS inhibitor, N_{ω}-Nitro-L-arginine methyl ester hydrochloride, (L-NAME) (Sigma, St. Louis, MO, USA) diluted in blood was fed to newly emerged flies (<24 hours post-emergence) (Figure 2.3 Panel D). Flies were then fed *T. b. brucei* BSFs infected blood 24 hours later at the second bloodmeal. Unfed flies were removed and the remaining flies were subsequently maintained on blood-spiked with L-NAME. Midguts were dissected 7 days post-infection (i.e adult flies were 10 days old) and scored for parasitaemia as described previously. The control group was fed blood mixed with the appropriate volume of sterile PBS. Four replicates were performed. However, a more appropriate negative control that should have been used is N(G)-nitro-D-arginine-methyl ester (D-NAME), the inert enantiomer of L-NAME (Ohno *et al.* 1994). This would have provided a more reliable result in comparing the active L-NAME to its inactive stereoisomer.

An initial titration experiment was performed to find the optimum concentration of the NOS substrate, L-Arginine (Sigma, St. Louis, MO, USA) needed to feed flies without causing significant mortality. Newly emerged flies (<24 hours old) were fed varying concentrations of L-Arginine mixed with blood (2 mg/ml, 10 mg/ml, 25 mg/ml and 50 mg/ml). Flies were then infected 48 hours later with *T. b. brucei* BSFs mixed with normal blood and unfed flies were removed. Flies were maintained on the same concentration of L-Arginine mixed with blood. Midguts were dissected 7 days later (i.e adult flies were 10 days old) and scored for trypanosome infection as previously described. The mortality rate of flies was recorded throughout the experiment.

2 mg/ml of L-Arginine was the optimum concentration chosen and was thus fed to newly emerged flies prior to infection with *T. b. brucei* BSFs 48 hours later (second bloodmeal). Unfed flies were removed. Flies were subsequently maintained on blood supplemented with L-Arginine until dissection at day 7 post-infection (i.e adult flies were 10 days old). Midguts were scored for trypanosome infection as previously described. Sterile PBS or 2 mg/ml L-Histidine spiked-blood were supplemented to flies and used as controls. The mortality rate of flies was recorded.

Similarly, an initial titration experiment of 1 mg/ml, 10 mg/ml and 20 mg/ml of a NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP) (InVitrogen, Eugene, OR, USA) were fed to newly emerged flies (<24 hours old) prior to infection with *T. b. brucei* BSFs 48 hours later. Flies were maintained on a SNAP-spiked bloodmeal diet until dissection 7 days later (i.e adult

flies were 10 days old) and midguts were scored for trypanosome infection. The mortality rate of flies was recorded throughout the experiment.

10 mg/ml of SNAP (optimum concentration chosen) was fed to newly emerged flies (<24 hours old) prior to infection with *T. b. brucei* BSFs 48 hours later (second bloodmeal). Unfed flies were removed. Flies were subsequently maintained on blood supplemented with SNAP until dissection at day 7 post-infection (i.e adult flies were 10 days old). Midguts were scored for trypanosome infection as previously described. Sterile PBS spiked-blood was supplemented to flies and used as control. The mortality rate of flies was recorded.

2.2.13 Salivary gland infections

Flies (12-24 hrs post-emergence) were fed BSFs GFP trypanosomes strain J10 (*T. b. brucei* MCRO/ZM/72/J10 CLONE 1) (Peacock *et al.* 2007) simultaneously with 1 mg/ml of L-NAME-spiked blood. Unfed flies were removed. Flies were then maintained on blood supplemented with L-NAME, and salivary glands were dissected 28 days after infection. Midguts were dissected into 100 µl of sterile PBS on a glass slide, while salivary glands were removed from the fly by slowly teasing them out of the thorax into a 200 µl sterile PBS drop. Midguts of flies were shredded into 100 µl of sterile PBS on a glass slide and infection status was scored by searching for motile trypanosomes in 10 random fields using dark field microscopy (125 X magnification). Infection status of both pairs of salivary glands was scored by searching for fluorescent trypanosomes in 10 random fields using a compound fluorescence microscope.

2.2.14 NOS activity measurement

NOS activity in tsetse tissues was measured using a Nitric Oxide Synthase Assay Kit (Calbiochem, Nottingham, UK), which is based on the biochemical conversion of [^{3}H] arginine to [^{3}H] citrulline via NOS. Midguts were dissected and flash frozen in liquid nitrogen and then stored at -80°C until use. Tissues were homogenized in 500 µl 1X homogenization buffer containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA and 1 mM EGTA, and centrifuged at 4°C for 5 min at 23,000 g. Supernatant was added (5 µl) to a 40 µl reaction mixture (50 mM Tris-HCl, pH 7.4, 6 µM tetrahydrobiopterin, 2 µM FAD, 2 µM FMN, 10 mM NADPH, 6 mM $CaCl_2$, 1 µl ^{3}H-Arginine (1µCi/µl) and H_2O) (PerkinElmer, Cambridge, UK). The reaction mixture was incubated at room temperature for 30 minutes and stopped by adding 400 µl stop buffer containing 50 mM HEPES, pH 5.5, and 5 mM EDTA. This was followed by the addition of 100 µl equilibrated resin to the samples, and centrifuged for 30

seconds at 12,000 g. NOS activity in each tissue was determined by adding 1 ml of scintillation fluid (Ultima Gold, PerkinElmer, MA, USA) and quantifying the radioactivity in the eluate using a 1450 MicroBeta TriLux liquid scintillation counter (PerkinElmer, MA, USA). NOS activity (counts per minute - CPM) was normalized against protein concentration as described in section 3.2.4.

2.2.15 Immunoblotting

Immunoblotting using Hybond^{TM}-P polyvinylidene difluoride (PVDF) transfer membrane (Amersham Biosciences, Amersham, UK) was performed as previously described (Beecroft *et al.* 1993). In brief, the primary antibody was diluted 1:500 in blocking buffer. The anti-uNOS polyclonal rabbit antiserum was raised against a region within 50 amino acids to the C-terminus of mouse iNOS and nNOS (ThermoScientific, IL, USA). The secondary antibody was a 1:10,000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG/IgM (Pierce, Woburn, MA, USA). The western blot was developed with SuperSignal Dura chemiluminescence substrate (Pierce Chemical Company, Rockford, IL) and Kodak Biomax MR film (Eastman Kodak Company, Rochester, NY, USA) was used to detect chemiluminescence. Proteins were stained on PVDF membrane with 0.2% (w/v) nigrosine in PBS. The exposed film was superimposed onto the stained PVDF membrane to reveal the precise location of the immunoreactive protein bands. The inducible NOS recombinant protein from mouse, expressed in *E. coli* was used as a control (Sigma, St. Louis, MO, USA) (Bredt and Snyder 1990).

2.2.16 Cytotoxicity Assay

The chromogenic/fluorogenic substrate alamarBlue^{TM} (Onyango *et al.* 2000) was used to perform minimal inhibitory concentration (MIC) assays on trypanosomes. Procyclic forms of healthy, log phase trypanosomes (*T. b. brucei* TSW196*, T. congolense* IL3000 and *T. b. brucei* YTat1.1*)* were adjusted to 2.0 x 10^4 cells/ml in fresh medium and 100 µl (2.0 x 10^3 cell/well) were added into round-bottom 96-well polypropylene microtitre plate wells (COSTAR/Corning Inc, Corning, NY, USA) containing serial dilutions (10 µl in MEM). After 72 hours of incubation at 27°C, 11 µl of 10X alamarBlue^{TM} (BioSource International, Inc., Camarillo, CA, USA) were added to each well and plates were incubated for another 6 h (final incubation of 72h). Cell free supernatant (70 µl) from each well was then transferred into 96-well flat-bottomed black/white microplates (Greiner Bio-One, CellStar, MJS Biolynx, Brockville, ON, Canada) for fluorescent measurement. Fluorescence was measured using a

Cytofluor 2300 microplate reader (Millipore, Bedford, MA, USA) at excitation and emission wavelengths of 540 nm and 590 nm respectively. The alamarBlue™ assay measures cell proliferation and viability based on the recognition of metabolic activity (Ahmed *et al.,* 1994). In response to chemical reduction by metabolically active cells, the alamarBlue™ colorimetric/fluorometric growth indicator which contains an oxidation-reduction indicator changes colour. Growth related reduction causes the indicator to change from oxidized (blue, non-fluorescent) to reduced (red, fluorescent). Trypanosomes were tested against L-NAME, L-Arginine and sodium nitroprusside dehydrate (SNP) (Sigma, St-Louis, MO, USA), an equivalent of SNAP. SNP was used instead of SNAP because of lack of availability of SNAP at the time of the experiment.

2.2.17 Bacterial NOS

Since NOS has been detected in Gram-negative bacteria, the possibility of the secondary tsetse endosymbiont, *Sodalis glossinidius* generating NOS was first investigated. SDS-PAGE/Western Blot was carried out on both a *Sodalis* pellet and supernatant from a *Sodalis* culture using the primary antibody, a polyclonal anti-universal NOS synthetic peptide as described in section 2.2.9. In addition, a range of *Sodalis* supernatants which were grown in different media were assayed for NOS activity as described in section 2.2.14.

We also investigated whether *Sodalis* elicits a NOS response from tsetse. Therefore, flies were fed a *Sodalis*-spiked bloodmeal and midguts of fed flies were dissected during a timecourse from 24 to 120 post-feeding. Midguts were dissected in 100 µl saline on a glass slide and placed into chilled Eppendorfs on ice. Subsequently, a NOS assay as described in section 2.2.14 was performed on the midguts.

2.2.18 Statistical analysis

Statistical analysis was performed using SPSS16 (SPSS Inc., Chicago, Illinois). Paired sample t-test was performed to determine if significant differences were present between two groups and ANOVA analysis was used to examine differences between more than two groups. Differences were significant if $p < 0.05$. A binary logistic regression was performed on the experiment involving early knockdown via injection of dsNOS, followed by infection with *T. congolense.* This was done to compensate for differences in blood composition (low haematocrit was noticed in blood aliquots fed to both experimental and control flies). Logistic regression fitted replicate and treatment and no effect of replicates was observed ($p>0.05$) and treatment was highly significant if $p<0.05$.

2.3 Results

2.3.1 Localization of NOS transcript expression in tsetse and other insects

The level of NOS transcript expression in tsetse was evaluated using a tissue survey performed by carefully dissecting different organs to check for localization of mRNA transcripts by semi-quantitative RT-PCR. NOS transcripts were detected in all tissues tested; these included midgut, salivary gland, fat body, reproductive tissue (testes), flight muscle and whole fly (Figure 2.4 Panel A). The transformed NOS clone (01b11) was used as a plasmid control (Figure 2.4 Panel B). The expression of NOS in midgut and salivary glands are particularly important as this is where the trypanosomes develop. In addition, the fat body is important as it is the major site for insect immunity (Hao *et al.* 2003; Lehane *et al.* 2008).

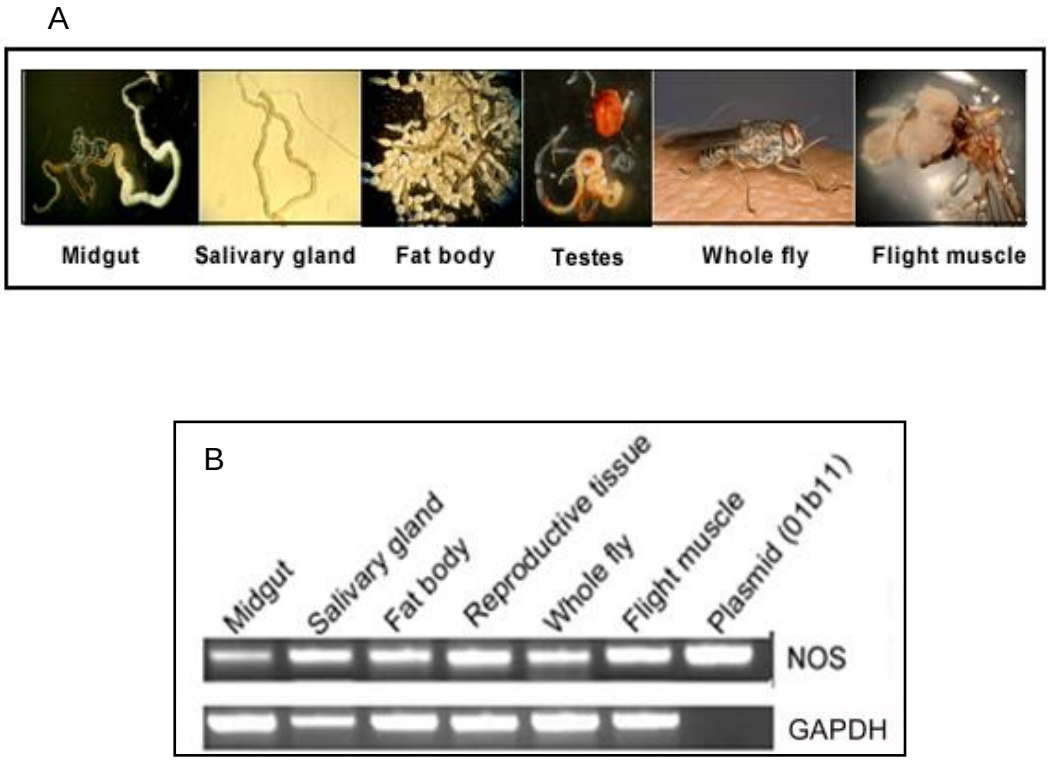


Figure 2.4. NOS transcript analysis in different tsetse tissues determined by semi-quantitative RT-PCR. (A) Photographs of different tsetse tissues: midgut, salivary gland, fat body, reproductive tissue (testes), whole fly and flight muscle. **(B)** Transcript profiles of different tissues probed with NOS and housekeeping (GAPDH) primers with a plasmid (01b11) as a positive control.

Only one NOS isoform has been characterized in insects and this isoform is believed to be inducible NOS (iNOS) because of its increased transcript and enzymatic expression in response to parasitic infections in several insect species (Dimopoulos *et al.* 1998; Luckhart *et al.* 1998; Whitten *et al.* 2001). To confirm that iNOS transcripts are expressed, DNA and RNA of cell lines of *An. gambiae* Sua 5.1 and *D. melanogaster*, together with a tsetse plasmid control were probed with NOS primers to check, as positive controls, for gene expression. NOS transcripts were indeed present in both *An. gambiae* and *D. melanogaster* cell lines (Figure 2.5 Panel A). Western analysis was also performed on midguts from *An. gambiae* and *G. m. morsitans.* The mosquito tissue was used as a positive control because Hillyer and Estevez-Lao (2010) successfully performed immunoblot analysis on *An. gambiae* using the same universal NOS antibody that recognizes a conserved region near the C-terminus of mouse iNOS and neuronal NOS (Figure 2.5 Panels B and C). However, neither the full length protein of mosquito NOS (160 kDa) nor the expected *G. m. morsitans* NOS (143 kDa) could be detected due to technical problems regarding the high percentage gel which limited the migration and hence detection of the high molecular weight proteins. Three immunoreactive bands were detected in both male and female *An. gambiae,* with approximate molecular weights of 55 kDa as detected, though as a minor band, by Hillyer *et al.* (2010) and two unknown bands of 27 and 13 kDa in size. A single copy of NOS exists in *An. stephensi,* however this gene is alternatively spliced into 18 to 22 distinct transcripts of which three are induced in the midgut following *Plasmodium* infections (Luckhart and Li 2001). The 55 kDa form of NOS could be a possible alternatively spliced variant since some of the splice variants of NOS in *An. stephensi* are predicted to be of approximately similar weight (Luckhart and Li 2001) and the *An. gambiae* NOS gene has one splice variant of similar weight. Exon-spanning PCR performed by Luckhart and Li (2001) revealed that alternative splicing of NOS mRNA contributed to diverse transcripts, however the numbers of splicing events in individual transcripts are unknown. Alternatively spliced mRNA transcripts of iNOS have also been detected in human epithelial cells, translating into three proteins with deletions in haem and FMN binding regions (Eissa *et al.* 1996). The 27 and 13 kDa bands, also detected in male and female tsetse, could be splice variants as the weight of one of the splice variant in *An. stephensi* is in the range of 13 kDa. The newly available *G. m. morsitans* genome suggests 16 splicing events generating 17 exons, though currently only one transcript isoform has been confirmed. However, multiple exon-specific probes are needed to confirm this. Alternatively, these two bands of 27 and 13 kDa might be NOS degradation products. These smaller bands might be a result of protein degradation by

proteases present in tsetse midgut. In addition, these bands could also result from bacterial proteins, as tsetse is known to harbor three symbionts in addition to diverse bacteria present in the midgut (Lindh and Lehane 2011). Therefore, feeding appropriate antibiotics to tsetse before dissection of midguts might help eliminate most bacteria present in the midgut. The expected size of *G. m. morsitans* NOS full length protein is 143 kDa, however the immunoblot analysis did not detect a 143 kDa band, possibly due to technical problems with the western analysis.

Hence, repeating the Western analysis with more appropriate conditions might allow for the detection of the full length protein and eliminate possible degradation products. In order to do so, first tsetse and mosquito midguts should be triturated in the presence of protease inhibitors to eliminate subsequent degradation of intact proteins by midgut proteases. In addition, a protein assay such a Bradford assay should be performed in order to standardize for the amount of protein being loaded per lane and in order to compare bands from tsetse and mosquito midgut samples. The samples can be reprobed with an antibody such as GAPDH in order to detect protein amounts, which would be expected to be equal in all samples. . Finally, a lower percentage gel such as an 8% gel would be more appropriate in detecting a 143 kDa band rather than a 12.5% gel. A low concentration gel of 8% or less would allow the large NOS protein to transfer out of the gel and migrate further through a lesser dense gel matrix, enabling separation of the bands.

2.3.2 Baseline NOS levels in unfed and fed flies

To first determine whether the presence of blood can influence NOS activity, a timecourse of teneral (newly emerged, unfed) versus age-matched fed flies was performed by collecting midguts every 24 hours up to 6 days post-emergence or post-feeding. Figure 2.6 depicts the NOS activity in teneral midguts over a 6 day timecourse. NOS activity is consistently higher in fed flies (solid line) than unfed flies (dashed line), however the same pattern of gradual increase in NOS activity is conserved by both groups of flies, with NOS activity peaking from 96 to 144 hours post-emergence.

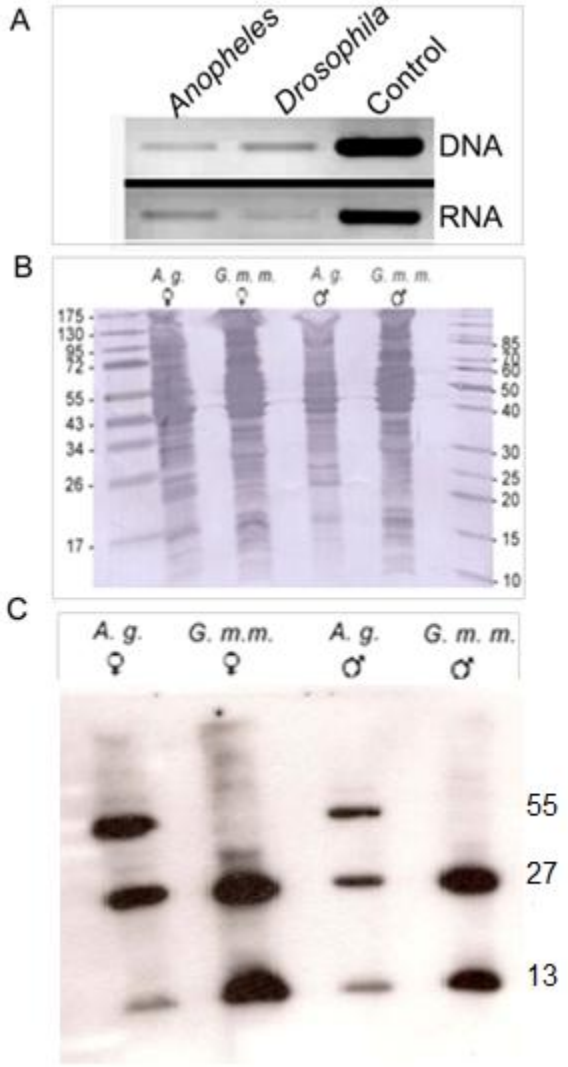


Figure 2.5. NOS transcript analysis in insect cell lines and immunoblot analysis on mosquito and tsetse midguts. (A) NOS transcript expression in DNA and RNA extracted from *An. gambiae* and *D. melanogaster* cell lines and plasmid (01b11) as a control. The RNA control is RNA extracted from tsetse midgut. **(B)** Individual midguts were solubilised in 2X Laemmli buffer and one midgut equivalent of either *An. gambiae (A. g.)* mosquito midgut or half of *G. m. morsitans (G. m. m.)* tsetse midgut was loaded onto each lane of a 12.5% polyacrylamide gel and blotted with a polyclonal anti-universal NOS antiserum. Ten kDa molecular mass ladder was used to determine immunoreactive band masses. PVDF membranes were stained with nigrosine (purple background) and **(C)** overlaid with developed film (one hour after exposure) to show precise location of protein bands and to ensure protein loading per lane.

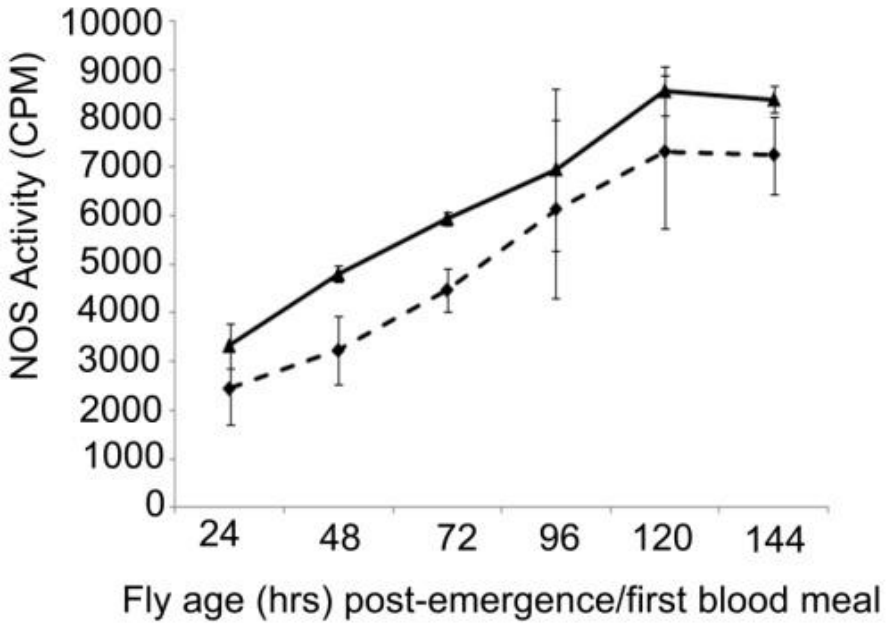


Figure 2.6. Temporal NOS levels in unfed and fed flies. Y-axis=NOS activity in counts per minute (CPM). Basal levels of NOS in unfed teneral flies (dashed line) and flies fed their first bloodmeal (solid black line) during a one to six day timecourse. N=180 for the entire experiment; mean ± SE for three biological replicates. Regression analysis for the timecourses indicate a p-value=0.664.

2.3.3 NOS is responsive to gene knockdown using RNA interference

RNA interference (RNAi) was employed to transiently reduce NOS mRNA transcripts in adult male tsetse flies. Preliminary injection experiments were conducted to first determine the optimal concentration of dsRNA needed to achieve maximum knockdown with minimal mortality rates due to wounding (Table 2.1 Panel A). Concentrations of dsRNA ranging from 1.5 μg to 6 μg were injected into flies and knockdown was examined in both midgut and reproductive tissue. Six μg of dsNOS was determined to be the optimal dsNOS concentration giving 68% transcript knockdown in both midguts and reproductive tissues and 0% mortality rates. Fly mortality was recorded 1 hour, 24 hours, 48 hours and 72 hours post-injection with 6 μg dsNOS (Table 2.1 Panel B) when mortality levels averaged ~20%.

An initial experiment was also performed to determine the duration of NOS transcript knockdown. Flies were injected with either 6 μg of dsNOS or 6 μg of dseGFP control. Comparison of NOS transcript expression by semi-quantitative RT-PCR analysis, normalized using GAPDH revealed that knockdown of NOS transcript peaked at day 5 post dsNOS injection (83.4% compared to dseGFP control: 8.6%) and then decreased up to day 13 post dsNOS injection (Figure 2.7 Panel A).

In order to confirm gene knockdown, different techniques were used to check transcript, protein and enzyme expression/activity following injection of dsNOS. Seven days after dsRNA injection, knockdown of NOS transcripts in midguts was 59.5% (relative transcript abundance: 40.5% ± 11.1) and reproductive tissues (testes) had 65.2% knockdown (relative transcript abundance: 34.8% ± 2.9) (Figure 2.7 Panel B). Due to high numbers of flies used (253 flies in total), wounded flies (flies pricked with a needle) were used to perform a comparative analysis of normalized densitometry with flies injected with dsNOS. The comparative analysis of normalized densitometry data also showed reduced transcript levels in dsNOS injected than a normal wounded fly (Figure 2.7 Panel B insert). At day seven post-dsRNA injection, the full length protein could not be detected in dsNOS injected, dseGFP injected and normal flies, however a reduction in the intensity of the 27 kDa band was detected by Western blotting, however due to unequal loading of the samples, we cannot conclude that protein knockdown occured (Figure 2.8 Panel A). Therefore confirmation of protein knockdown via Western analysis is inconclusive and needs repeating under appropriate gel conditions to ensure detection of the full length protein. A NOS assay which measures endogenous NOS activity (Figure 2.8 Panel B)

showed reduced NOS activity by approximately 28% in dsNOS injected flies compared to dseGFP injected and normal flies, however this was not significant. Hence more replicates are needed to determine whether there is a small but significant difference in enzymatic activity following dsRNA injection.

Table 2.1. Transcript knockdown and mortality rates with different concentrations of dsNOS. (A) Male adult flies were injected with varying concentrations of dsRNA (1.5 µg, 3 µg, 4.5 µg and 6 µg). NOS transcript knockdown (7 days post-injection) in midgut and reproductive tissue was measured via RT-PCR. The % transcript knockdown is highlighted in grey and % fly mortality observed in each group was recorded in the bottom half of each box. **(B)** Observed mortality rates after three replicates of injections with optimal concentration of 6 µg of dsNOS 1 hr, 24 hrs, 48 hrs and 72 hrs post-injection. N=number of flies per group.

A

	1.5 µg	**3 µg**	**4.5 µg**	**6 µg**
Midgut	48.5 % 0% n=10	55.7 % 20% n=10	60.3 % 0% n=10	67.7 % 0% n=10
Reproductive Tissue	34.2% 0% n=10	41.8% 20% n=10	63.2% 0% n=10	67.6% 0% n=10

B

	n	**1 hr**	**24 hrs**	**48 hrs**	**72 hrs**
Rep 1	25	0%	0%	0%	8%
Rep 2	105	1.9%	4.8%	20%	21.9%
Rep 3	131	2.3%	5.3%	19.8%	22.9%

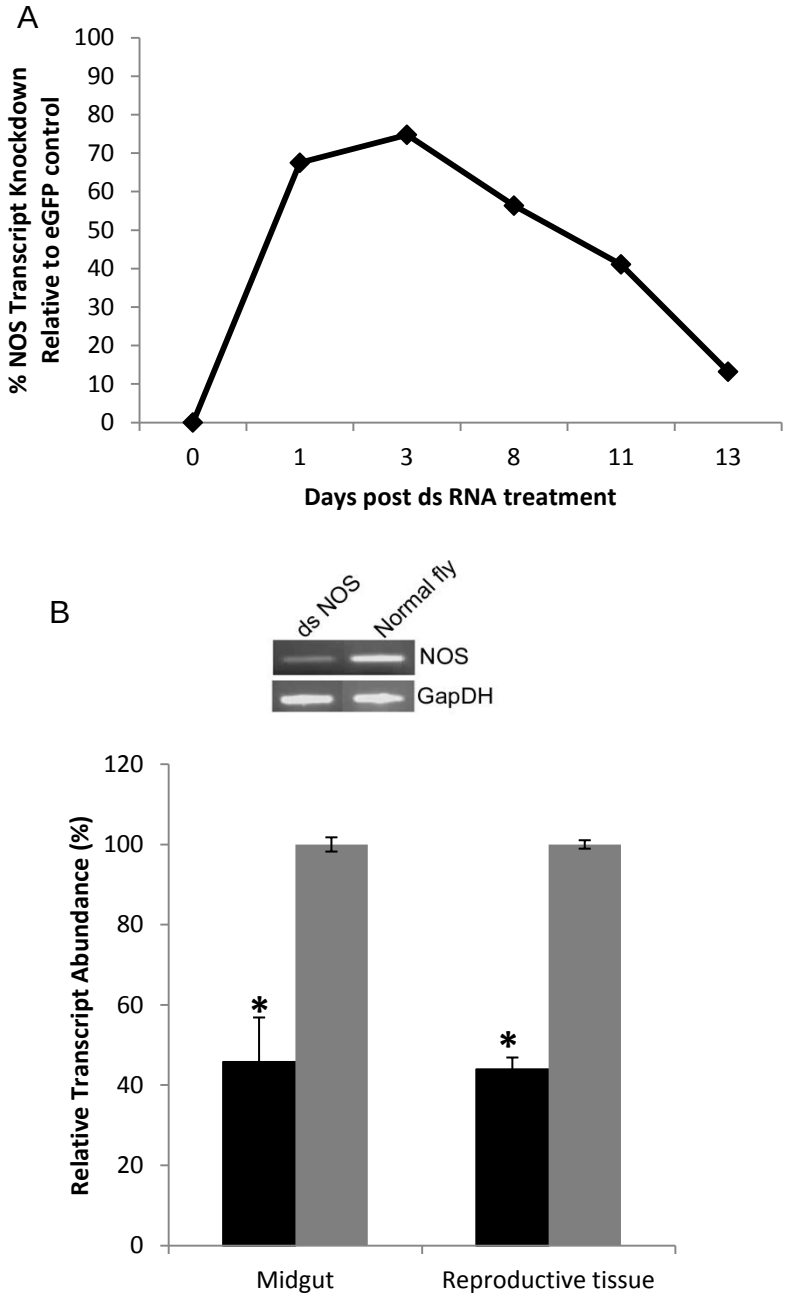


Figure 2.7. Semi-quantitative RT-PCR analysis of NOS expression following injection of dsNOS and the gene knockdown of tsetse NOS (using RNA interference) was confirmed by a reduction in NOS transcript seven days after the injection of dsRNA. (A) Tsetse were injected with 6µg dsNOS (solid black line) or a control dseGFP (dotted black line). Data are presented as the mean knockdown (%) of NOS transcript (y-axis) plotted against the number of days after injection of dsRNA (x-axis). Band intensities were normalized separately for each lane using the corresponding GAPDH loading control band intensities. Each data point represents the average of five flies. **(B)** Knockdown efficiency on day seven post-dsNOS in tsetse midguts and testes as determined by densitometry from RT-PCR, normalized against the housekeeping gene, GAPDH; black: dsNOS injected, grey: wounded control; midgut p-value (*): 0.037; reproductive tissue p-value (*): 0.008 with student's t-test; n=253 (total number of flies for the experiment); mean ± SE for three biological replicates. Insert: NOS transcript abundance was significantly reduced in the midgut as determined by semi-quantitative RT-PCR.

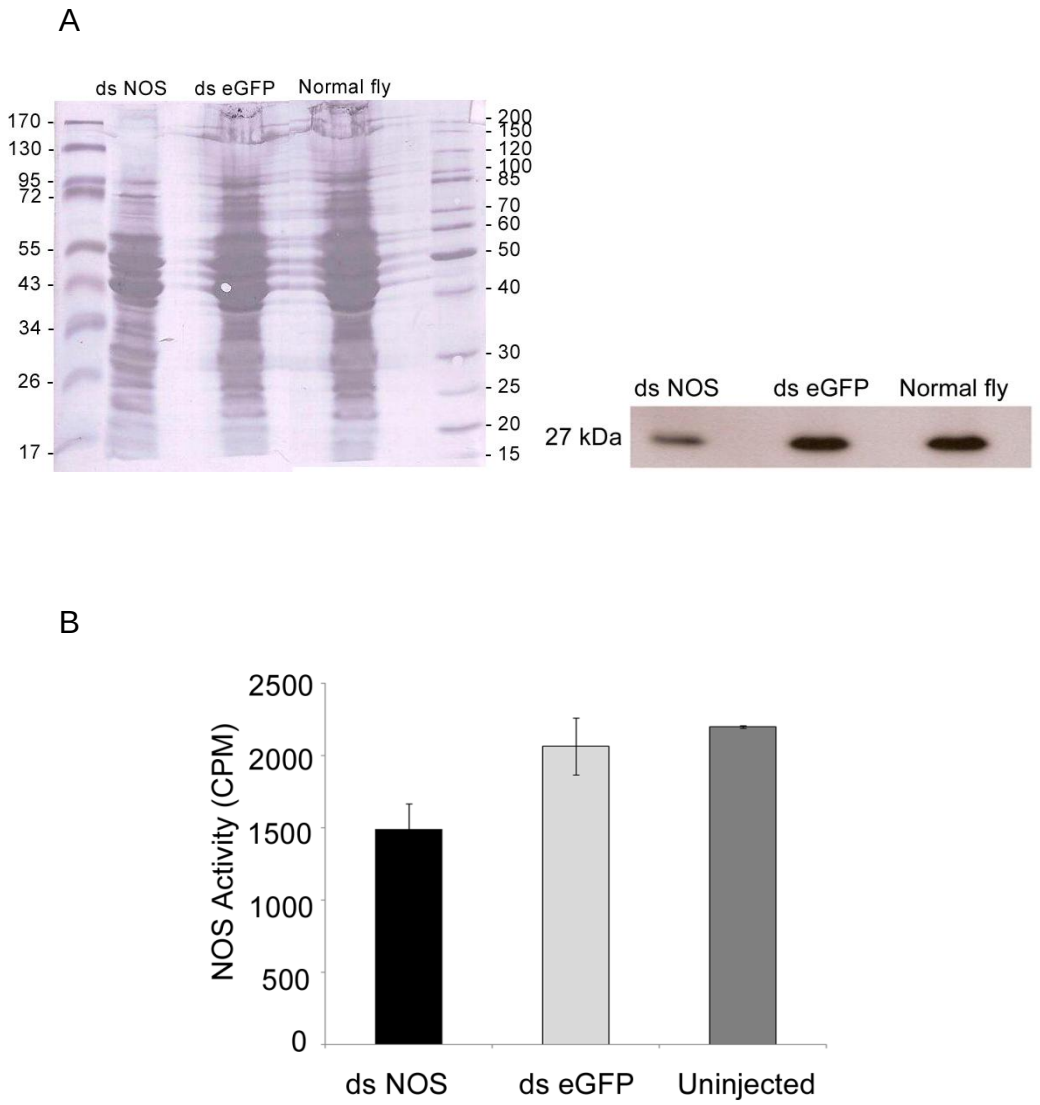


Figure 2.8. Western analysis and NOS enzyme activity seven days after the injection of dsRNA.. (A) Western blot using an anti-uNOS polyclonal antibody showing a less intense 27 kDa band compared to ds eGFP injected and normal flies. Left: PVDF membrane was stained with nigrosine. Right: developed film (one hour after exposure) to show precise location of protein bands and to ensure protein loading per lane. **(B)** NOS activity was reduced in flies injected with dsNOS compared to control groups. N=40; total number of flies assayed for all three groups. Mean ± SE for two biological replicates.

2.3.4 Effects of gene silencing on parasite prevalence

The effect of knockdown of NOS activity was monitored prior to (early) and after (late) arrival of trypanosomes in the tsetse midgut. By thoracically injecting dsNOS **after** the first bloodmeal, NOS levels were knocked down (via RNAi) prior to flies receiving parasite-infected second bloodmeal (*T. b. brucei* TSW196 BSF). The reduction of NOS pre-infection led to a two-fold increase in midgut infection prevalence (50.6% ± 1.73) compared to the controls, which were significantly lower (dseGFP = 25.5% ± 2.87 and uninjected group = 25.1% ± 5.45) (Figure 2.9 Panel A). Differences were significant with p-values (ANOVA) being < 0.001 (uninjected control and dsNOS) and < 0.001 (dseGFP control and dsNOS). The parasite prevalence in the control groups are consistent with typical midgut infection rates observed with an infectious second bloodmeal using *T. b. brucei* (TSW196) (Haines *et al.* 2010). Flies injected with dsNOS were twice as susceptible to midgut infections compared to control groups.

The experiment was repeated using another economically important species of parasite, *T. congolense* and a similar trend was observed. Depletion of NOS from the fly midgut prior to infection of the flies led to a 1.8 fold increase in *T. congolense* midgut infections (34.8% ± 3.14) compared to both control groups (dseGFP= 21.4% ± 3.86 and uninjected control= 19.4% ± 3.86) (Figure 2.9 Panel B). A binary logistic regression was used to compare differences in order to compensate for the low haematocrit observed in blood fed to experimental and control flies in two of the three replicates. This was done in order to correct for the large variances which would arise, particularly after data from the three biological replicates were pooled together. Large variances would have accounted for large error bars and significance values between treatment groups would be more likely reflect differences in replicates due to low haematocrit rather than differences between treatment groups. The total number of flies dissected for this experiment was 290, however the dseGFP injected control experienced high mortality rates with n=59 compared to n=120 and n=111 for uninjected control and dsNOS injected respectively.

Late knockdown of NOS transcript was accomplished by infecting male flies in the first bloodmeal (*T. b. brucei,* TSW196) and then injecting them with dsNOS 24 hours later. Midguts were dissected seven days later. Transcript repression of NOS led to 29.5% ± 2.11 midgut infection prevalence, while controls were lower (dseGFP=20.6% ± 2.93 and uninjected group=10.1% ± 2.66) (Figure 2.9 Panel C). Although NOS levels were reduced, differences between dsNOS and uninjected control was significant (p-value < 0.01) while a

statistically insignificant midgut infection prevalence (p-value > 0.05) between dseGFP control and dsNOS was observed. This implies that reduction of NOS expression after the parasites arrive in the midgut has little effect on parasite establishment in the midgut. Hence, the antioxidant environment within the tsetse midgut as the trypanosomes arrive in it is the most important issue for parasite survival.

The average midgut infection prevalence at the 5th bloodmeal is typically less than 5% (Haines *et al.* 2010). Therefore, midgut infection prevalence was examined in these flies which are deemed to be refractory. As before, NOS transcripts were repressed by injecting dsNOS after the first bloodmeal. However, flies were fed regular blood until the 5th bloodmeal, where flies were fed a trypanosome infected bloodmeal instead. Midguts were dissected seven days later and scored for infection prevalence (Figure 2.9 Panel D) which revealed that the uninjected group (5.4% ± 3.21) and dseGFP group (3.17% ± 3.2) had infection rates consistent with expected midgut infection prevalence at 5th bloodmeal. Midgut infection prevalence in dsNOS experimental group was higher (19.1% ± 5.64) than controls (approximately five times). However, differences were not significant between experimental dsNOS and dseGFP injected (p-value > 0.05) while differences between dsNOS and uninjected control were significant (p-value < 0.05). Flies were dissected 14 days post-dsNOS injection, suggesting that the knockdown effect is likely diminishing (15.2% transcript knockdown of NOS compared to ~2% transcript knockdown dseGFP control at day 13 post-dsRNA injection (Figure 2.7 Panel A)). Hence we might expect variability in the flies, and the larger variances make it difficult to see significant differences.

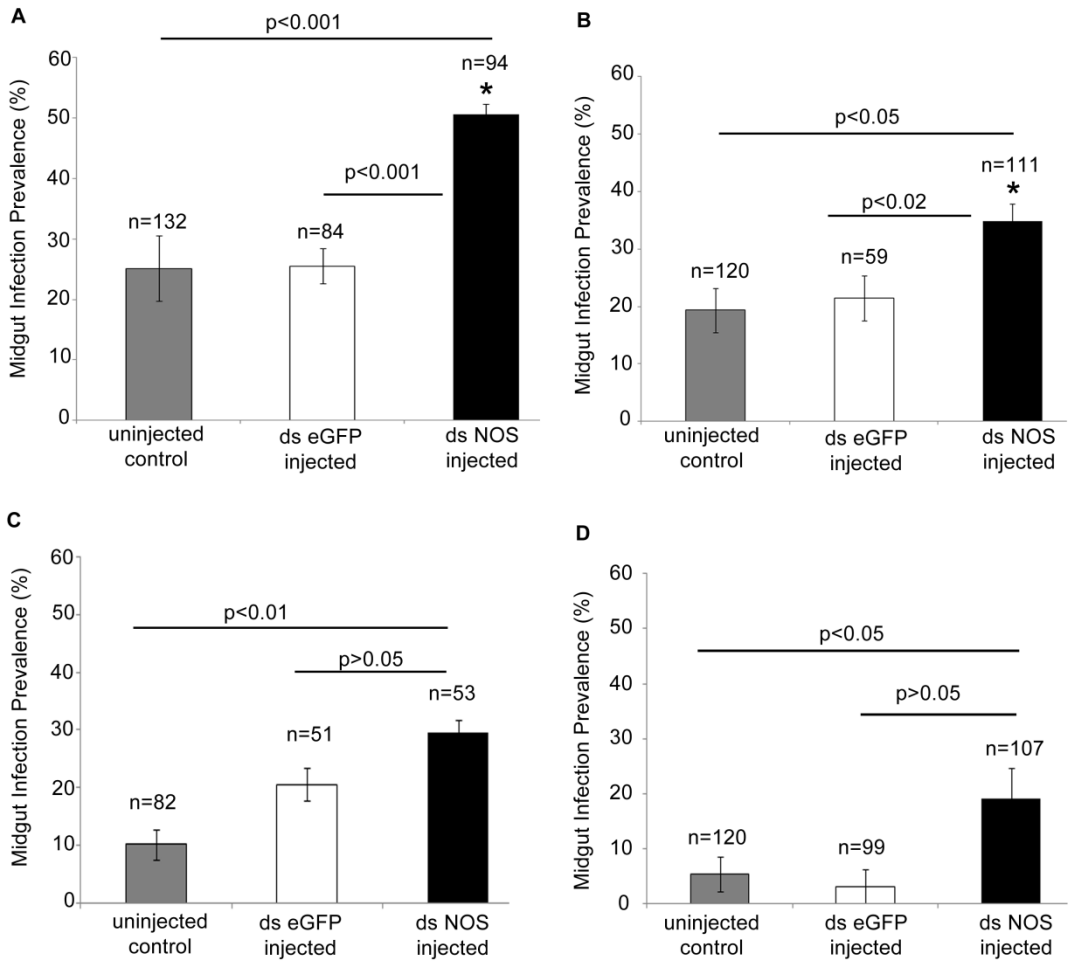


Figure 2.9. Parasite prevalence in the tsetse midgut before and after NOS knockdown. **(A)** Early NOS repression: flies were injected with dsNOS prior to feeding on *T. b. brucei* infected blood; * = p-value of <0.001 between the two injected groups. N=310 (total number of flies for the experiment). **(B)** Early NOS repression where flies were injected with dsRNA prior to feeding on *T. congolense* infected blood; * = p-value of <0.02 between the two injected groups. N=290 (total number of flies for the experiment). **(C)** Late NOS repression: flies were first fed an infected bloodmeal (*T. b. brucei*) then injected with dsNOS. N=186 (total number of flies for the experiment). **(D)** Flies injected with dsNOS after the 1st bloodmeal and infected with *T. b. brucei* at 5th bloodmeal. N=326 (total number of flies for the experiment). Mean ± SE for three biological replicates. Logistic regression fitted replicate and treatment and no effect of replicates was observed (p>0.05) and treatment was highly significant if p<0.05 using ANOVA.

2.3.5 Dietary manipulation of NOS levels and the consequences for trypanosome infection *in vivo*

To confirm whether exogenous repression of NOS by feeding flies NOS inhibitors would give similar results to our RNAi observations, flies were fed N^G-Methyl-L-arginine acetate, (L-NMMA). However, due to high fly mortality rates (93-98%) observed (Figure 2.10 Panel A), an alternative NOS inhibitor was selected. Oral administration of a NOS inhibitor N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) spiked into the bloodmeal resulted in an increase in *Plasmodium berghei* oocyst numbers in infected mosquitoes (Luckhart *et al.* 1998). To determine if trypanosome establishment can be likewise affected, 1 mg/ml of L-NAME inhibitor was continuously fed to tsetse flies upon emergence, with the only exception being omission from the infectious bloodmeal. The dietary supplementation of L-NAME resulted in a significantly higher (2.3 fold increase, p-value < 0.001) midgut infection prevalence (37.4% ± 5.86) compared to the PBS control (15.9% ± 3.64) (Figure 2.10 Panel B). The difference in mortality rate between the L-NAME group (21.5% ± 5.1) and PBS control group (12.9% ± 2.4) was not statistically significant (Figure 2.10 Panel A). The intensity of midgut infection (%) was also recorded but there was no difference between infected control and L-NAME flies which were highly infected with > 100 parasites per field (Figure 2.10 Panel C).

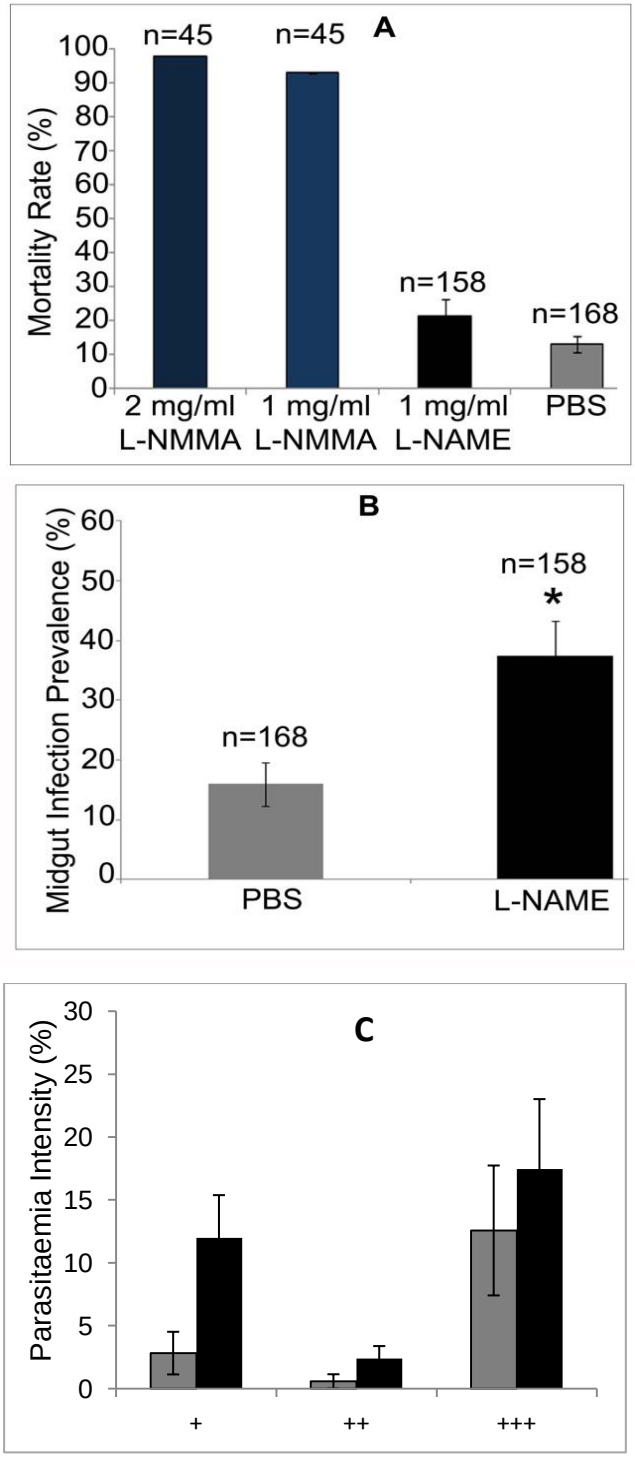


Figure 2.10. NOS inhibition using N^G-Methyl-L-arginine acetate, (L-NMMA) and N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME). **(A)** L-NMMA and L-NAME induced mortality rates (%) seven days post-feeding. **(B)** Flies were first fed L-NAME prior to receiving the infected second bloodmeal *(T. b. brucei)*, and then further maintained on an L-NAME diet until day 7 post-infection (black) or control flies fed on steady diet of PBS and normal blood (grey); mean ± SE for four biological replicates. Midgut infection prevalence in control and L-NAME fed flies on day 7 post-infection; * = p-value <0.001. **(C)** Overall parasitaemia intensity (%) for PBS (grey) and L-NAME (black) fed flies. + = <10 parasites/field; ++ = 11-99 parasites/field; +++ = >100 parasites/field.

L-Arginine, a NOS substrate was also included as a dietary supplement. Since L-Arginine acts as a NOS substrate, the addition of L-Arginine in the diet could result in elevated NOS levels, and a subsequent reduction of midgut infections due to a more hostile midgut environment. An initial titration experiment was conducted using varying concentrations of L-Arginine. Mortality rates (Figure 2.11 Panel A) did not show a clear trend; 50 mg/ml supplementation of L-Arginine resulted in an 18% midgut infection prevalence with 0% mortality and 25 mg/ml dietary L-Arginine caused a 7.1% midgut infection prevalence with 21.4% mortality (white bar in Figure 2.11 Panel A). The remaining concentrations of 10 mg/ml and 2 mg/ml induced no mortality and 21.4% and 15.4% midgut infection prevalence was observed respectively.

Since the results from this experiment showed no clear trend, we selected to feed 2 mg/ml of L-Arginine as the optimal concentration based on previous literature (Hao *et al.* 2003) and low fly mortality. Two feeding controls were used, PBS and L-Histidine (another polar amino acid) that is not involved in the NOS pathway (Figure 2.11 Panel B). The differences in midgut infection rates between the L-Arginine group and control groups were not significant (p-value > 0.05 between L-Arginine and L-Histidine; p-value > 0.05 between L-Arginine and PBS). Mortality rates were also similar across the three treatment groups and not significant (p-value > 0.05 between L-Arginine and L-Histidine; p-value > 0.05 between L-Arginine and PBS).

Another NOS donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Ali *et al.* 2011) was also tested for its effect on midgut infection prevalence. An initial titration experiment was conducted (Figure 2.11 Panel C) to determine the optimum concentration of SNAP to use that would not cause high mortality rates. Flies that fed on SNAP had a significantly higher midgut infection prevalence (71% ± 12.4) compared to PBS control (21.5% ± 6.7) with a p-value of 0.044. This gave the opposite result to that expected from the hypothesis (Figure 2.10 Panel D). Mortality rates between SNAP fed flies (21.1 ± 5.65) and PBS control flies (13.7 ± 3.7) were not significant (p-value = 0.165). This was not pursued further.

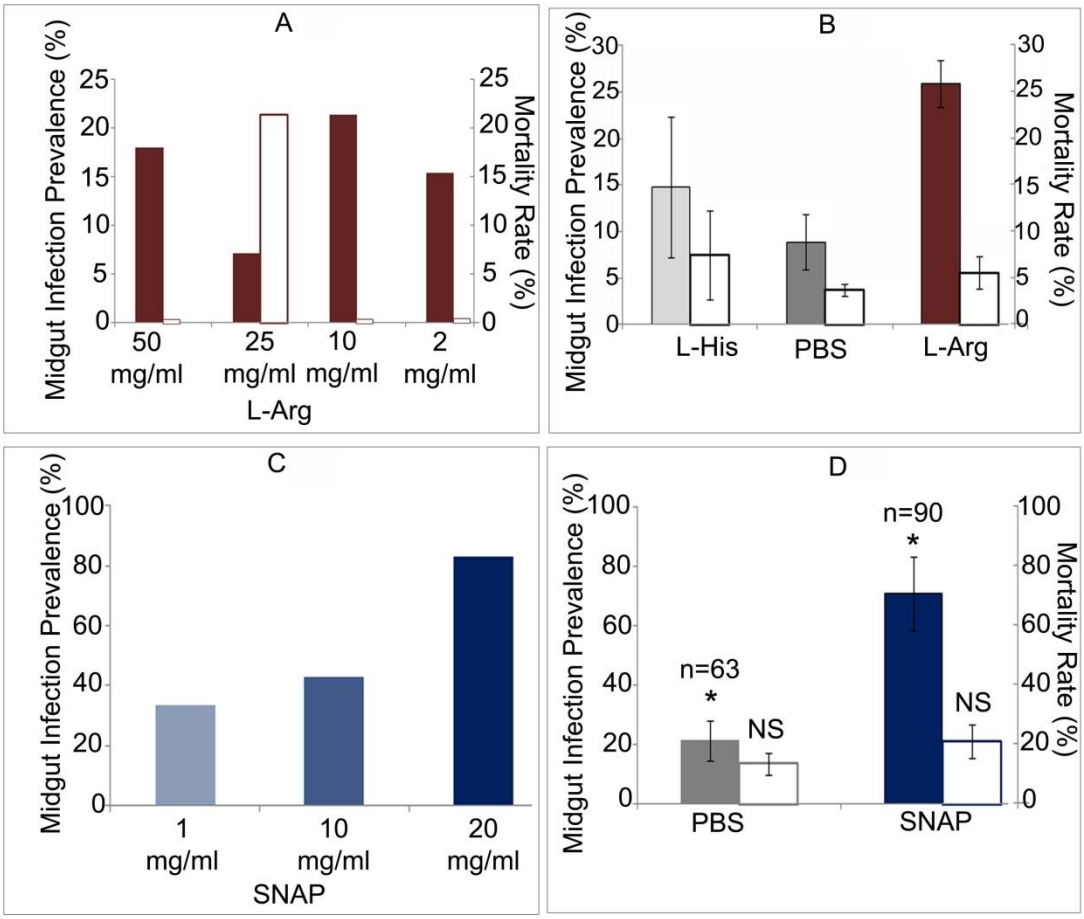


Figure 2.11. Dietary supplementation of NOS substrate, L-Arginine and NOS precursor, *S*-nitroso-*N*-acetylpenicillamine (SNAP). (A) Titration experiment of different concentrations of L-Arginine, midgut infection prevalence (solid red bars) and mortality rates (%) in solid white bars. Mortality rates for 50, 10 and 2 mg/ml are 0%. **(B)** Midgut infection prevalence (%) of flies fed on 2 mg/ml of L-Arginine versus PBS and 2 mg/ml of L-Histidine. White bars represent mortality rates (%). **(C)** Titration experiment on the effect of different SNAP concentrations on midgut infection prevalence (%). **(D)** Midgut infection prevalence (%) of flies fed on 20 mg/ml of SNAP versus PBS control in solid grey bars; flies infected with *T. b. brucei* at second bloodmeal; * = p-value of 0.044. Mortality rates (%) in solid white bars. NS: not statistically significant between the two groups; mean ± SE for three biological replicates.

2.3.6 *In vitro* cytotoxicity assays

To determine if dietary supplementation of the bloodmeal with NOS inhibitor or precursor had an indirect effect on the parasites themselves, several *in vitro* Minimal Inhibitory Concentration (MIC) assays were performed using three species of trypanosomes. This assay uses the chromogenic substrate alamarBlue™ which, when metabolized by living cells, is reduced and changes from blue to pink. The growth of all procyclic trypanosomes was inhibited by high concentrations of L-NAME. The lab reference strain, *T. b. brucei* TSW196 ceased to grow in concentrations of L-NAME exceeding 0.14 mg/ml. *T. congolense* IL3000 ceased to grow in 0.28 mg/ml of L-NAME and *T. b. brucei* YTat1.1 in concentrations of 1.14 mg/ml. The calculated concentration of L-NAME in the bloodmeal is 0.99 mg/ml. In contrast, even the highest concentration of L-Arginine, the comparative positive control, had no negative effects on procyclic metabolism and growth. Both L-Arginine (a NOS precursor) and SNP (a NOS donor) were not trypanotoxic to *in vitro* cultured procyclic trypanosomes at concentrations 5.0 mg/ml and 0.30 mg/ml respectively.

2.3.7 NOS expression following bacterial challenge *in vivo*

To determine if tsetse use NOS to respond to bacterial infections, flies were orally-challenged with bacteria. Flies were fed either Gram-negative (live or dead *E.coli* K12 RM148 (OD_{600}=0.5)), dead (live is lethal to flies (Needham *et al.* 2004)) Gram-positive bacteria (*S. aureus* SH1000 (OD_{600}=0.5)) or LPS-free PBS mixed with normal blood. The flies were dissected every 24 hours over a five day timecourse. Surprisingly, no differences in NOS activity was observed between bacteria infected midguts and the PBS control, which suggests that the ingested live or dead bacteria we assayed does not elicit a change in natural NOS levels (Figure 2.12 Panel A). The same gradual increase in NOS activity is observed in most of the groups. An immunoblot analysis was also performed on midguts of flies injected with *E. coli* versus a normal wounded fly (Figure 2.12 Panel B). Western blot analysis confirms that *E. coli* does not elicit a NOS response since band intensities are similar in both sets of flies.

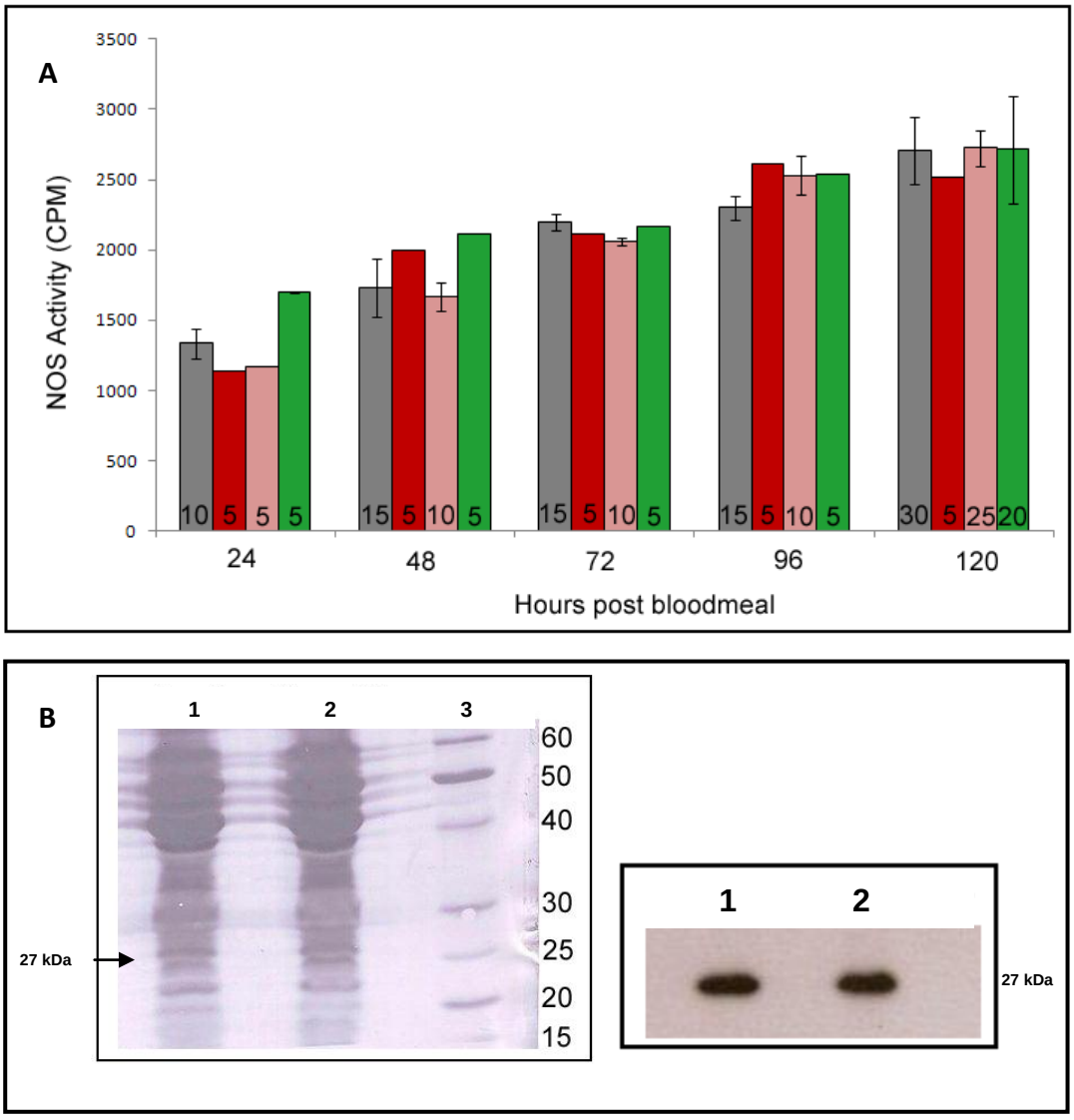


Figure 2.12. NOS expression via NOS activity assay and immunoblot analysis following bacterial challenge. (A) NOS activity (CPM) in flies every 24 hours up to 120 hours post feeding with either PBS (grey), live *E. coli* (red), dead *E. coli* (pink) or dead *S. aureus* (green). Mean ± SE for three biological replicates with the exception at 120 hours where mean ± SE for six biological replicates. Total number of flies assayed is represented in individual bars in bar graph. **(B)** Immunoblot analysis in midguts from dead *E. coli* injected flies (lane 1) or normal wounded fly (lane 2). Individual midguts were loaded onto each lane of a 10% polyacrylamide gel and blotted with a polyclonal anti-universal NOS antiserum. PVDF membranes were stained with nigrosine (purple background) and the film was developed one hour after exposure to show precise location of protein bands and to ensure protein loading per lane. Lane 3 = 10 kDa ladder (Page Ruler).

2.3.8 NOS activity following parasitic challenge *in vivo*

Flies were fed trypanosome infected blood (*T. b. brucei* TSW196) in the second bloodmeal and midguts from batches of flies were dissected two days and six days post-infection and assayed for NOS activity. Figure 2.13 Panel A shows that NOS activity increases in flies two days post-infection (weakly-infected flies: 2003 CPM ± 164 and highly-infected flies: 2302 CPM ± 120) compared to naive flies (1775 CPM ± 305) that were not exposed to parasites. NOS activity continues to increase slightly in flies with low infections (10-99 parasites/field) and significantly increases in flies with soaring infections (> 100 parasites/field). At day six post-infection, flies with high infection (> 100 parasites/field) have high NOS activity (3125 CPM ± 224), and flies with very few trypanosomes in the midgut (many are self-cured by day six since no trypanosomes were detected via microscopy) had NOS activity (2507 CPM ± 219) equivalent to uninfected flies (2420 CPM ± 299). Differences between highly infected flies at day six and self-cured flies/weakly infected flies are significant, with a p-value < 0.02. These data suggest that NOS is naturally up regulated upon trypanosome infection.

A comparative experiment was also done to check NOS activity in flies infected with bacteria (dead *E. coli* and *S. aureus)* compared to flies infected with *T. b. brucei* at day five post-infection (Figure 2.13 Panel B). Again, NOS activity was not statistically different (p-value > 0.05 between *E. coli* and PBS and p-value > 0.05 between *S. aureus* and PBS) between flies infected with bacteria *(E. coli:* 3056 CPM ± 297; *S. aureus:* 3129 CPM ± 401) compared to control, LPS-free PBS group (3188 CPM ± 397). However, NOS activity was statistically different (p-value < 0.02) in flies with low infections (7099 CPM ± 799) compared to flies with high infections (4257 CPM ± 491), the latter value is similar to bacteria infected flies. Figure 2.13 Panel C superimposes NOS activity in low (between 10 and 99 parasites/field) and high (> 100 parasites/field) infected flies at days two, five and six post-infection.

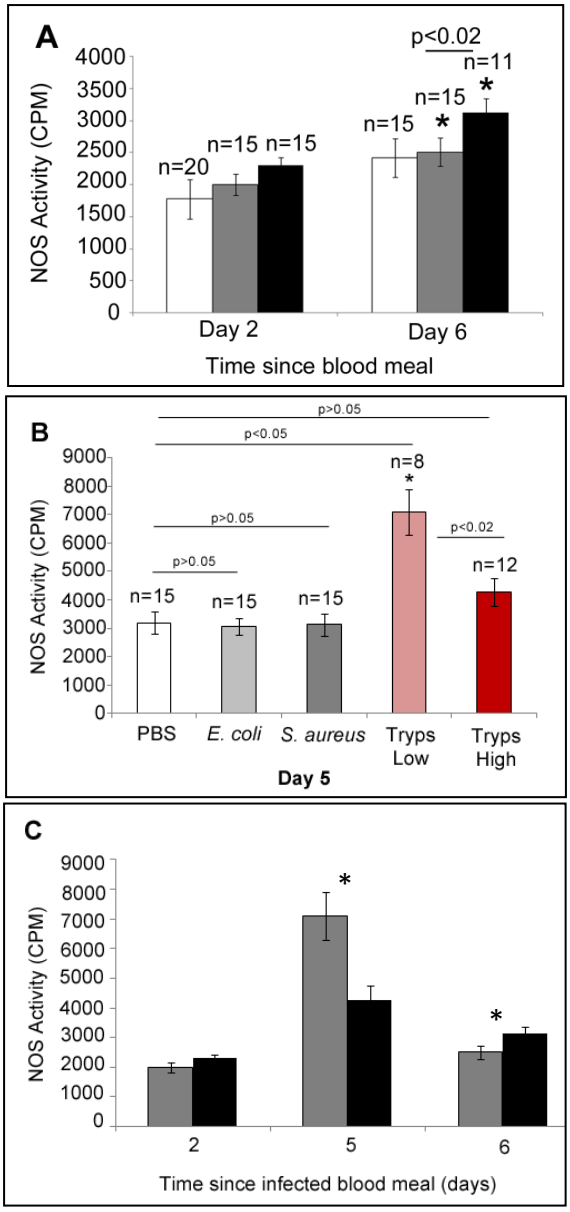


Figure 2.13. NOS activity in midguts from flies fed bloodstream form trypanosomes with low (10-99 parasites/field) or high (>100 parasites/field) infections. (A) NOS activity (CPM) in days two and six post-infection. White bars = naive, dark grey bars = low infection, black bars = high infection. P-value (*) <0.02 between infected flies (high and low) at day six. **(B)** NOS activity (CPM) in flies exposed to PBS (white), dead *E. coli* (light grey), dead *S. aureus* (dark grey), and flies exposed to trypanosomes with flies with low trypanosome infection (pink) and red bar = high infection at day five post-infection. P-value (*) <0.02 between low and high parasite infections. **(C)** NOS activity of flies with low infections (grey) and high infections (black) at days two, five and six post-infection. Mean ± SE for three biological replicates A, B and C. P-values (*) <0.02.

2.3.9 Salivary gland infections

Trypanosome transmission to the mammalian host is via saliva of an infected tsetse fly. Standard salivary gland infection prevalence was determined by infecting flies with *T. b. brucei* MCRO/ZM/72/J10 CLONE 1 bloodstream forms and dissecting 28 days later. These GFP-expressing trypanosomes were monitored using microscopy luminescence/photography. First bloodmeal midgut infection prevalence was 57% while salivary gland infection prevalence was 12% (Figure 2.14 Panel A). This indicates that 19.4% of the midgut infections matured into salivary gland infections. In our lab, we typically observe salivary gland infections from 10 - 15%. Once the average salivary gland infection rate for our system was established, teneral infected flies were maintained on an L-NAME diet. Flies were dissected 28 days post-infection and scored for midgut and salivary gland infection prevalence (Figure 2.14 Panel B). All infected salivary glands had both pairs of glands infected (Figure 2.14 Panel C).

Midgut infection prevalence of flies fed on L-NAME were slightly higher (58.95% ± 8.95) compared to flies fed PBS (48.15% ± 15.15) or normal blood (51.9%). However there was no difference in salivary gland infections between flies fed on L-NAME (2.65% ± 2.65) compared to flies fed on PBS only (2.65% ± 2.65). Due to no differences observed in salivary gland infections between L-NAME and PBS fed flies, no further experiments were carried out to check for salivary gland infections.

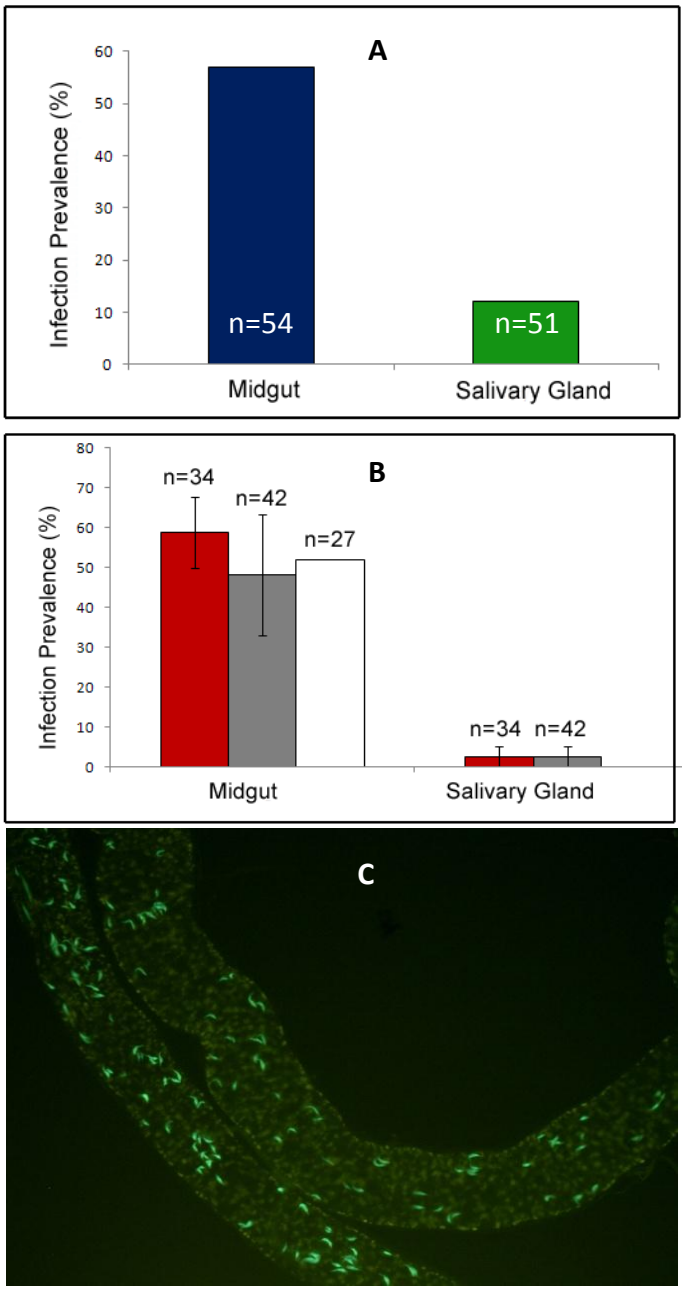


Figure 2.14. Expression of GFP-labelled trypanosomes in midgut and salivary glands 28 days post-infection. (A) Basal levels of infection prevalence (%) in midguts and salivary glands of flies fed GFP-labelled trypanosomes and maintained on sterile blood. **(B)** Infection prevalence (%) in midguts and salivary glands of flies fed on an L-NAME supplemented diet. Red bar: L-NAME supplemented bloodmeal, grey bar: PBS control, white bar: flies fed on normal sterile blood. Mean ± SE for three biological replicates, n=103 (total flies) for the entire experiment. **(C)** Fluorescent trypanosomes within a pair of infected salivary glands.

2.3.10 NOS levels between tsetse sexes do not differ significantly

To ascertain if NOS levels differ between male and female flies, we assayed midguts from age matched (<6 hours old) teneral males and females. NOS levels observed in male midguts were higher (7513.83 CPM ± 1861) than in female midguts (4530 CPM ± 1657), although this difference was not significant (p-value = 0.148) (Figure 2.15 Panel A). Interestingly, the NOS measurements in these teneral male midguts are high when compared to previously observed baseline levels of NOS in teneral midguts (24 hrs: 2452 CPM ± 740). Mean ± SE is for three biological replicates (n=19 total).

2.3.11 NOS levels in tsetse midguts are not influenced by the temperature at which the puparia were maintained

Emerging male flies were collected from puparia reared at either 26°C (insectary) or 30°C (incubator). Midguts from flies less than 24 hours old were dissected. Figure 2.15 Panel B shows increased NOS activity in midguts of flies that have emerged from puparia kept at 26°C (7514 CPM ± 1861) compared to midguts from emerging male flies kept at 30°C (4611 CPM ± 675). Although decreased NOS activity is observed in flies emerging from heat-shocked puparia (equated to high susceptibility (Burtt 1946)), differences were non-significant (p-value = 0.37). With mean ± SE from three biological replicates, error bars are large, most likely due to the small sample size (n=21 total). Best attempts were made to obtain age-matched flies (typically less than six hours old). No visual physical differences were observed between flies from the two treatment groups. In both cases, females emerged earlier than males (two to three days earlier), and flies emerging from puparia kept at 30°C emerged approximately a week earlier than flies emerging from puparia kept at 26°C.

2.3.12 *Sodalis glossinidius* does not induce NOS levels

The possibility of the secondary tsetse endosymbiont, *Sodalis glossinidius* generating NOS was first investigated. The *S. glossinidius* genome was screened and 31% amino acid homology was found to the tsetse NOS reductase domain. The *S. glossinidius* pellet and supernatant were screened with the anti-universal NOS antibody by immunoblotting, however the results were inconclusive. In addition, *S. glossinidius* supernatants grown in different media were screened using a NOS assay, however the results were also inconclusive. Thus, due to these uncertain results we cannot confirm whether *Sodalis* produces NOS.

Next, we investigated whether *Sodalis* elicits a NOS response from tsetse. Figure 2.15 Panel C depicts NOS levels in midguts of flies fed dead *Sodalis*-spiked bloodmeal compared to NOS levels in midguts of flies fed dead *E. coli,* dead *S. aureus* and PBS. We observed increasing NOS levels from 24 to 120 hours in all treatment groups except midguts of flies fed *Sodalis* (black bar) which appeared to have suppressed NOS levels (24 hrs: 536 CPM) compared to the PBS control (24 hrs: 1337 CPM). NOS levels in the midguts of *Sodalis* fed flies were typically around 679 CPM compared to the other treatment groups which were all relatively higher, and continued to increase every 24 hours post-feeding. This suggests that *Sodalis* results in a suppressed NOS response from the tsetse fly. However more replicates are needed in order to statistically confirm this.

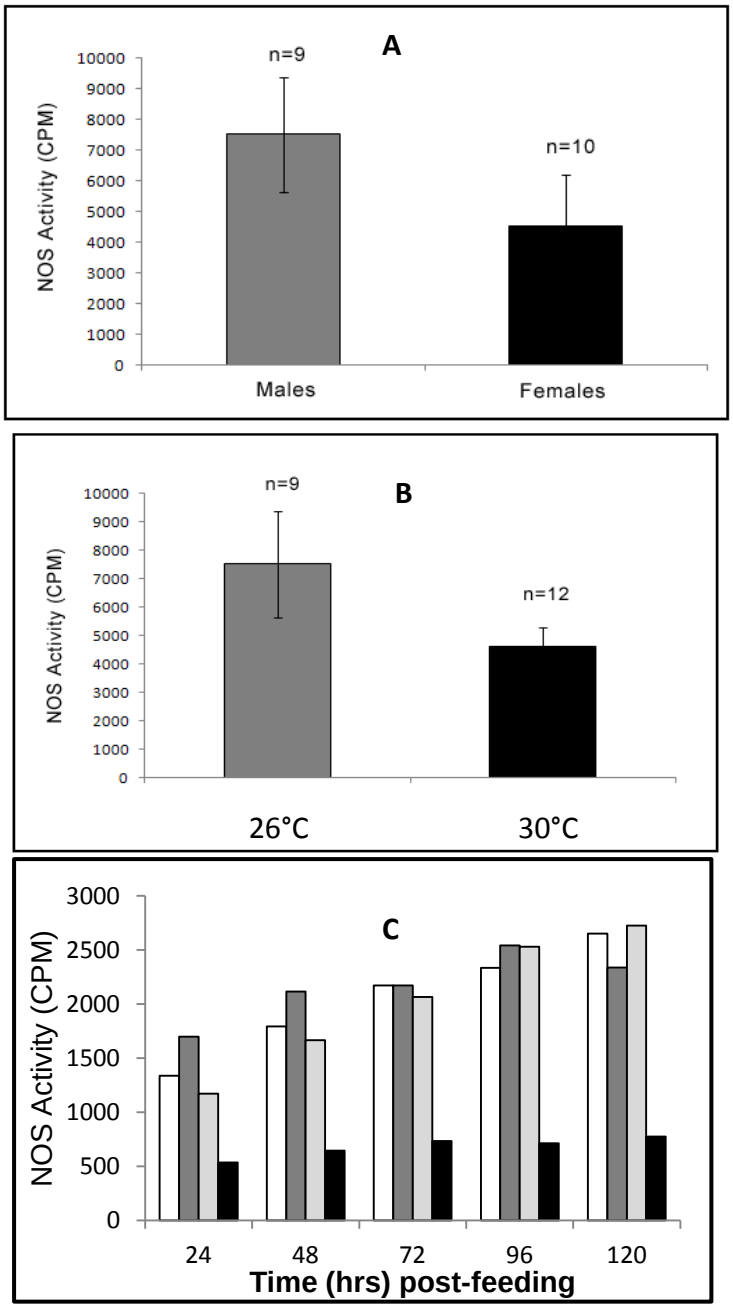


Figure 2.15. NOS activity (CPM) in midguts of male and female flies and male flies emerging from puparia reared at 26°C and 30°C. (A) NOS activity in midguts of age-matched newly emerged male (grey) and female (black) *G. m. morsitans* (<6 hours old). Mean ± SE for three biological replicates. **(B)** NOS activity in midguts of male flies (<6 hours old) emerging from puparia kept at either 26°C or 30°C until emergence. Mean ± SE for three biological replicates. **(C)** Timecourse of NOS activity (counts per minute - CPM) at 24, 48, 72, 96 and 120 hours post-feeding in midguts of tsetse fed with *S. glossinidius* (cells and supernatant; in black), dead *E. coli* (dark grey), dead *S. aureus* (light grey) and PBS control (white). One replicate only.

2.4 Discussion

Most vector insects are able to eliminate pathogens due to a strong innate immune system (Lemaitre and Hoffmann 2007; Davis and Engstrom 2012). The innate immune system of tsetse has been linked to refractoriness of tsetse to trypanosome transmission (Leak 1999; Hao *et al.* 2001; Hao *et al.* 2003; Hu and Aksoy 2006). This chapter examines whether NOS acts as one of the barriers to trypanosome establishment in the tsetse midgut.

We first investigated changes in NOS expression following feeding and observed that NOS activity was higher in older flies than younger flies. The increased NOS activity in flies following a bloodmeal is consistent with earlier reports in insects (Hao *et al.* 2003; Ali *et al.* 2011). Increased NOS activity was observed in tsetse proventriculus 24 hours post-feeding (Hao *et al.* 2003) while in mosquito midgut lumen, haemoglobin released from bloodmeal digestion catalyzes the synthesis of nitric oxide (NO) metabolites in a reducing environment leading to the generation of additional reactive nitrogen species (RNS) (Peterson *et al.* 2007). Although the NOS activity profile was conserved in both fed and unfed flies, NOS activity peaked as flies entered starvation (generally seven days for an adult fly and three to four days for a teneral fly) (Kubi *et al.* 2006) which is the time they are most likely to be taking a subsequent bloodmeal. Starvation can deplete energy reserves leading to fluctuations in NOS levels due to a distressed immune response.

There is strong evidence to suggest that NOS and NO are involved in response to parasitic infections in a range of species. Nitric oxide has also been shown to inhibit growth and multiplication of *T. congolense* in mice (Lu *et al.* 2011). In mouse macrophages NO acts as a potent molecule, causing cell death of intracellular *Leishmania major* parasites (Zangger *et al.* 2002; Nahrevanian *et al.* 2009). In the triatomine bug, *Rhodnius prolixus,* NO regulates *T. rangeli* infections and interferes with the *T. cruzi* life cycle *in vivo* and *in vitro* (Vespa *et al.* 1994; Whitten *et al.* 2001). In addition, NO has been shown to trigger apoptosis in *P. berghei* ookinetes (Ali *et al.* 2011). Expression of NOS transcripts are higher in mosquitoes infected with *Plasmodium* (Dimopoulos *et al.* 1998; Luckhart *et al.* 1998) and manipulation of NOS via inhibitors and donors affect the susceptibility of mosquitoes to *Plasmodium* infections (Herrera-Ortiz *et al.* 2004; Herrera-Ortiz *et al.* 2011).

In this study, it is demonstrated that NOS levels increase following trypanosome infection. An increase in NOS production/activity in infected midguts demonstrates an up regulation of NOS in response to live trypanosomes. An increased NOS activity at day five in flies with

low parasitaemia (many of these flies had no parasites, therefore are deemed 'self-cured') was first observed. Gibson and Bailey (2003) have shown that at day five, some trypanosomes, associated with or imbedded in the peritrophic matrix, are cyst-like in appearance possibly due to degenerating forms. Perhaps NOS levels are induced as an increasing amount of trypanosomes are being lysed at day five. Of greater interest is that at day six, midguts of self-cured tsetse flies have similar NOS activities to those from 'naive' uninfected midguts, which indicate that NOS is indeed up regulated by an active trypanosome infection. This suggests that perhaps at day five, lysis of large numbers of trypanosomes induce NOS, and by day six, in established infections (high parasitaemia), basal NOS levels are maintained when these trypanosomes are not being lysed and therefore the NOS pathway is not activated.

Furthermore, reducing levels of NOS, before the infectious bloodmeal is taken, permits an increased number of flies to become infected with both *T. b. brucei* and *T. congolense*. RNAi-induced knockdown of NOS or feeding of L-NAME prior to infection leads to decreased production of NOS and NO, and when parasites arrive with the bloodmeal, the NO-depleted environment enhances their establishment (up to 2 fold increase in infection prevalence). It would appear that NOS levels at the early stages of the infection process are particularly important because knockdown of NOS 24 hours after infection has a lower impact on trypanosome prevalence than early knockdown. However, when NOS is repressed post parasite arrival (via RNAi), the prevalence remains unaffected, thus suggesting that the initial midgut environment plays a vital role in the success or failure of parasite establishment in the fly. Our results do not agree with those of Hao *et al.* (2003), who also explored the possible role of NOS in tsetse trypanosome interactions by using dietary supplementation with L-NAME (Hao *et al.* 2003). They report no difference in parasite prevalence in tsetse and concluded that NOS and NO did not play a significant role in trypanosome midgut establishment (Hao *et al.* 2003). The difference arises possibly because these authors first infected flies with trypanosomes before adding L-NAME to the diet at subsequent bloodmeals. Consequently they will have missed the critical period early in the infection process.

The use of D-enantiomers of NAME have been used in several studies as an inactive control for NOS inhibition (Luckhart *et al.* 1998; Foley and O'Farrell 2003; Herrera-Ortiz *et al.* 2004; Ali *et al.* 2011). L-NAME competes with L-Arginine for the site of action of the enzyme while D-NAME is inactive and cannot compete with L-Arginine leading to the latter binding

to the active site (Alderton *et al.* 2001). Subsequently, L-Arginine acts as a substrate for NOS leading to NO production. In hindsight, D-NAME should have been used as a negative control instead of PBS alone because using the same buffer such as PBS to dilute both L-NAME (active) and D-NAME (inactive) would have provided a more reliable comparison, discounting any differences such as the pH value between L-NAME and controls.

Dietary supplementation of the NOS substrate L-Arginine had no effect on trypanosome prevalence and this may be because L-Arginine is already in excess in the fly. In addition, perhaps NOS is the rate limiting step in the reaction of L-Arginine converting to L-Citrulline and NO. Therefore, the addition of L-Arginine might have no effect, but rather maybe the addition of NOS itself would lead to an increase in NO, if in fact, NOS is the rate limiting step. The addition of SNAP, a NO donor resulted in the opposite phenotype to that expected from our hypothesis. SNAP spontaneously disintegrates into NO disulfide, however how SNAP functions in blood and in an alkaline pH environment of tsetse midgut is unknown. The half-life of SNAP is typically around 4.6 hours in an aqueous solution (Ignarro *et al.* 1981). Flies were infected with trypanosomes after a SNAP diet, suggesting that levels of NO are highest in the tsetse midgut when the parasites are ingested and enter the midgut environment. Our hypothesis suggests that the reduction of NO results in an increase in parasites. Therefore, likewise feeding SNAP should result in increased NO levels, and increased NO in the tsetse midgut should inhibit parasite establishment, therefore a decrease in parasite prevalence is expected. However, feeding of SNAP resulted in an increase in parasites in the midgut. Thus if SNAP is indeed increasing NO levels in the tsetse midgut and increasing parasite prevalence, then this indicates that the initial hypothesis is incorrect. Therefore, levels of NO need to be measured after the addition of SNAP to the diet, to check whether it is indirectly causing an increase in NO levels. The Greiss reaction can compare the production of NO in the form of nitrite by SNP and SNAP (Ali *et al.* 2011) or a NO microsensor can be used to study the decomposition of SNAP as previous data suggests a 60% conversion efficiency of SNAP to NO at 20°C (Carton *et al.* 2009).

In addition, trypanosomes may possess a strong antioxidant defense mechanism such as trypanothione, an antioxidant enzyme which might suppress increased NO levels in the presence of SNAP. Since SNP was not toxic to trypanosomes *in vitro,* this hints at a strong defense response present in trypanosomes to protect against high levels of NO. However,

this again hints at rejecting the hypothesis that high NO levels results in decreased parasite prevalence.

Although transcript knockdown of NOS resulted in increased parasites, reduction in NOS activity and protein knockdown following dsRNA injection were inconclusive. In addition, although feeding L-NAME, known to reduce NO, resulted in increased midgut parasite prevalence, the appropriate negative control D-NAME was not used. Ali *et al.* (2011) showed that SNP induced significant increase in apoptotic ookinetes *in vitro,* suggesting that enhancing the NO production by *Plasmodium* kills ookinetes while L-NAME which reduces NO, led to significant decrease in the proportion of apoptotic ookinetes *in vitro.* Similarly, Hererra-Ortiz *et al.* (2004) have shown that *P. berghei* ookinetes exposed to SNP are killed. *In vivo,* SNAP resulted in decreased midgut infection prevalence while L-NAME supplemented diet resulted in a significant increase in *P. herghei* oocysts developing in the midgut. D-NAME, the inactive isomer had no effect (Ali *et al.* 2011). The *in vivo* results are similar to those found by Luckhart *et al.* (1998) who confirmed that *An. stephensi* limits *Plasmodium* development with NO by demonstrating that dietary provision of L-NAME increased parasite numbers while dietary provision of L-Arginine, the NOS substrate, reduced *Plasmodium* infections. Thus in order to demonstrate that tsetse limit trypanosome establishment with NO, we would expect SNAP to decrease parasite prevalence and L-NAME to increase parasite prevalence in the tsetse midgut.

No differences in parasite infections in salivary glands of flies fed either L-NAME or PBS were found. One possible explanation for the lack of phenotype in salivary glands could be that not enough L-NAME is reaching the salivary glands to compensate for an excess of NOS. If time permitted it would have been interesting to inject L-NAME into the fly and look again at the two phenotypes because under these circumstances we might expect L-NAME concentration in salivary and midgut tissues to be comparable. The *in vitro* parasite toxicity to L-NAME is likely due to the inhospitable pH of the L-NAME stock solution (pH 3.0) combined with the poor buffering capacity of the assay medium. When equal volumes of L-NAME solution were added to horse blood, the bloodmeal remained at neutral pH because of the high buffering capacity of blood, suggesting that this is not a problem with *in vivo* experiments. This supports the observation that when L-NAME was continuously spiked into the bloodmeal and fed to tsetse, there was an increase in parasite midgut prevalence and parasitaemia, rather than a decrease which one would expect if L-NAME itself were indeed trypanotoxic. Thus, we conclude that the ingested trypanosomes were

not damaged by the continuous administration of L-NAME in the bloodmeal. Both L-Arginine and SNP were not trypanotoxic to *in vitro* cultured trypanosomes.

NOS and NO have been found to be involved in response to bacterial infections in various organisms. For example, decreased NOS levels led to increased susceptibility of *Drosophila* larvae and adults to both septic and natural infections with Gram-negative bacteria (Foley and O'Farrell 2003). The induction of NOS in response to bacterial challenge has been shown in the moth, *Manduca sexta* (Eleftherianos *et al.* 2009) and in mosquitoes (Hillyer and Estevez-Lao 2010). By contrast, in tsetse NOS levels did not change in response to bacterial infection with both Gram-negative *E. coli* and Gram-positive *S. aureus*. Immunoblot analysis confirmed that injection of *E. coli* into tsetse did not elicit a NOS response although it is known that it does elicit other innate immunity responses which induce resistance to trypanosome infections (Hao *et al.* 2001). Our results differ from Hao *et al.* (2003) since they observed a significant increase in NOS activity 24 hours after flies were infected with dead *E. coli* (via ingestion) of a different strain. A possible explanation could be that Hao *et al.* (2003) measured NOS activity in the proventriculus of tsetse, while our study measures NOS activity in the entire tsetse midgut. While this includes the proventriculus this only forms a small proportion (<5%) of the total mass of midgut tissue. It appears that NOS activity differs in different sections of the tsetse alimentary canal or perhaps different strains of *E. coli* elicit a different immune response.

In addition, an explanation for the unexpected observations between tsetse NOS response and other insects' NOS response to bacterial infections may lie in the feeding pattern of tsetse flies compared to mosquitoes and *Drosophila*. *Drosophila* have mixed diets and they often ingest large quantities of micro-organisms. In contrast, tsetse feed exclusively on sterile vertebrate blood. It is known that the immune response in insects is energetically costly (Aksoy *et al.* 1997; Moret and Schmid-Hempel 2001; Ahmed *et al.* 2002; Armitage *et al.* 2003; Mallon *et al.* 2003; Hu *et al.* 2008; Catalan *et al.* 2011). It is feasible that tsetse flies, with their highly specialised diet, may be able to avoid using this part of the immune response. Another possible reason for these unexpected findings may lie in the obligate symbionts on which tsetse rely for nutritional supplements (Aksoy *et al.* 1997). Without the symbionts, fly fecundity and longevity are drastically reduced (Aksoy 2000; Pais *et al.* 2008). *Wigglesworthia* is housed in the midgut while *Sodalis* also has a high presence there. Consequently a strong NOS response to bacteria may be inimical to them. This all suggests considerable further work on this interesting phenomenon.

Tsetse contain several species of bacteria and we investigated whether the relative abundant Gram-negative symbiont, *S. glossinidius* (closely related to *E. coli*) elicits a NOS response from tsetse. Suppressed NOS levels (via NOS assay) were observed in flies fed *S. glossinidius* compared to flies fed *E. coli, S. aureus* and PBS, however due to one replicate performed only, this experiment warrants more replicates to significantly confirm the differences. There have been suggestions that *Sodalis* may influence tsetse vector competence. *S. glossinidius* has been shown to favour trypanosome establishment in tsetse midgut (Maudlin *et al.* 1990; Dale and Welburn 2001). The reduced NOS levels elicited by tsetse fed *Sodalis*-spiked bloodmeal might explain the increased trypanosome prevalence in tsetse midgut or perhaps tsetse is immunotolerant to the symbiont and does not mount an immune response.

To determine whether temperature and sex, both known to influence parasite susceptibility, might be due to NOS, NOS activity was measured in flies exposed to different rearing conditions. Previous reports indicate that puparia of *G. m. morsitans* kept at a higher temperature produce more parasite-susceptible flies (Burtt 1946). Although midguts of flies emerging from puparia kept at 26°C had higher NOS levels (more resistant) than midguts of emerging flies from puparia kept at 30°C (more susceptible), differences were not significant. There is published data that suggests that male flies mature more trypanosome infections than female flies (Milligan *et al.* 1995). However with our *G. m. morsitans* colony and the strains of trypanosome we are using, female flies are more susceptible to *T. b. brucei* and *T. congolense* infections than male flies (Walshe *et al.* 2011). Consequently it is interesting to note that we observed higher NOS levels in midguts of male flies although differences between the sexes were not significant. Perhaps larger sample sizes would provide more precise differences in NOS levels if NOS was involved in temperature or sex-dependent susceptibility.

In order to elucidate protein function within a biological system, some of the successful ways are to observe the mutant phenotype of an organism that lacks a gene of interest or to target a gene's product by introducing homologous dsRNA. Although the knockdown of the tsetse NOS gene has been confirmed at transcript level and a resulting phenotype was observed, the NOS assay indicates no significant reduction in NOS activity. In addition, the NOS assay may measure other isoforms of NOS (if there are tsetse homologs) as the assay simply measures the conversion of L-Arginine to L-Citrulline. The results from the SDS-PAGE/Western Blot were also inconclusive. The primary antibody, a polyclonal anti-

universal NOS synthetic peptide corresponding to a region of 50 amino acids to the C-terminus of mouse iNOS and nNOS has not been previously tested against tsetse NOS and therefore may recognize other isoforms of NOS if present in the tsetse. In addition, the 143 kDa band corresponding to the NOS protein was not detected, but instead, smaller molecular weight bands were detected. Hence although a reduction in band intensity of the 27 kDa band was observed, this band needs to sequenced in order to validate whether it is a splice variant of NOS, a degradation product resulting from tsetse midgut proteases or a bacterial contaminant. Therefore use of protease inhibitors when tsetse midguts are being processed, in addition to loading the samples on a lower percentage gel should result in detection or lack of the full length protein following injection of dsNOS. However, it should be noted that as other isoforms of NOS, particularly nNOS, have sequence homology to the only available tsetse NOS sequence, it is possible that the Western analysis may detect the other NOS isoforms, if present in tsetse.

In conclusion, RNAi-induced knockdown of NOS at transcript level and dietary supplementation of a NOS inhibitor are associated with an increased the frequency of trypanosome infection by 2 fold. In addition, the greatest effect is seen if these changes are brought about before trypanosomes enter the midgut. However, dietary provision of SNAP, a NO donor also increased trypanosome prevalence in the tsetse midgut, suggesting that the role NO in controlling trypanosome establishment is not proven and requires further study. This data however would indicate that the pre-existing midgut environment is crucial to whether parasites are able to establish themselves.

CHAPTER 3

The effect of reactive oxygen species (ROS) on trypanosome establishment in the tsetse fly

3.1 Introduction

The generation of reactive oxygen species (ROS) in mammals is an immediate epithelial response to pathogens (Fang 2004). Most vector insects are able to clear pathogenic agents due to a robust innate immune system. The mechanisms behind insect immune responses vary from phagocytosis, to activation of proteolytic cascades such as melanisation, production of antimicrobial peptides and production of ROS (Dimopoulos *et al.* 2001). In vertebrates, respiratory bursts activated by macrophages include the production of superoxide anion ($O_2^{-\bullet}$) by NADPH oxidase and transformed to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), leading to an increase in ROS. Myeloperoxidase then uses H_2O_2 as a substrate to produce hypochlorous acid (HOCl), a highly bactericidal compound (Fang 2004; Molina-Cruz *et al.* 2008).

In recent years, an increasing number of research groups have revealed the importance of ROS in combating parasitic and bacterial infections in insects. In *Drosophila,* ROS are produced at basal levels by the NADPH oxidase enzyme, dual oxidase (Duox) (Ha *et al.* 2005a; Ha *et al.* 2005b). However, activation of the epithelial immune system upon ingestion of bacteria results in increased ROS production (Ha *et al.* 2005a; Ha *et al.* 2005b).

In *Anopheles gambiae* mosquitoes, ROS play a role against both bacterial and parasitic infections (Molina-Cruz *et al.* 2008; Cirimotich *et al.* 2011). Strains of *An. gambiae* with high systemic ROS levels experience higher survival rates upon bacterial infections, while supplementation of antioxidants in the mosquito's diet resulted in lower survival rates when exposed to bacteria (Molina-Cruz *et al.* 2008). In addition, *An. gambiae* with high ROS levels result in *Plasmodium* killing in the midgut, rendering the mosquito refractory to *Plasmodium* infections (Kumar *et al.* 2003). Likewise, administration of uric acid, an antioxidant, decreases mortality after *P. berghei* infection, suggesting that oxidative stress can be dangerous to infected mosquitoes (Molina-Cruz *et al.* 2008).

ROS has been shown to activate a cell death pathway in *T. b. brucei* procyclics (Ridgley *et al.* 1999; Wang *et al.* 2002). In tsetse flies, the presence of bloodstream form trypanosomes induce H_2O_2 levels in the proventriculus, suggesting that ROS might be involved in clearing trypanosomes early in the infection process either directly or indirectly through the activation of other immune gene products involved in fly refractoriness (Hao *et al.* 2003). Furthermore, indirect evidence suggests that antioxidants detoxify free radicals in the tsetse midgut promoting trypanosome survival and establishment (Macleod *et al.* 2007b).

Supplementation of antioxidants such as glutathione, cysteine, *N*-acetyl-cysteine, ascorbic acid and uric acid in the infective bloodmeal increases trypanosome infection rates in the tsetse midgut (Macleod *et al.* 2007b). Likewise, increasing H_2O_2 levels via catalase knockdown results in decreased number of *P. berghei* in *An. gambiae* midguts (Molina-Cruz *et al.* 2008). However, there is no direct evidence to show that an increase in H_2O_2 levels in tsetse midgut results in a decrease in trypanosome prevalence.

The efficacy of transmitting parasites varies widely between the different tsetse fly species (Leak 1999; Aksoy *et al.* 2002). In general, Palpalis group are poorer vectors of *T. congolense* compared to the Morsitans group (Harley and Wilson 1968; Moloo and Kutuza 1988) while the latter are poorer vectors of *T. b. gambiense* than the Palpalis group (Richner *et al.* 1988). In laboratory conditions, transmission rates of infected flies vary between 1 and 20% dependent of fly species and parasite strain (Van den Abbeele *et al.* 1999; Gibson and Bailey 2003). Typically, less than 1 to 5% of collected field populations of tsetse flies are infected with *T. brucei* spp. complex (Woolhouse *et al.* 1994; Lehane *et al.* 2000). Upon closer examination of the prevalence of trypanosomes in natural tsetse populations, we realize that tsetse flies may mount a significant level of immune defence as observed infection rates in the field are low.

An indirect approach was used to test whether a positive correlation exists between midgut ROS levels and resistance to trypanosomes. Typically, trypanosomes replicate for the first 3 days following the infected bloodmeal; however after this time, complete eradication of infection occurs in a proportion of flies through an attrition phase (Gibson and Bailey 2003). Scoring the outcome of midgut infection can only be achieved 6-8 days after infection (Gibson and Bailey 2003). At this time, fly midguts either contain live parasites (can be seen microscopically) or no parasites (these flies had an infected bloodmeal but the attrition phase led to elimination of parasites, and thus these flies are deemed 'self-cured'). Although we are interested in determining whether ROS are involved in eliminating trypanosomes, we can only determine whether a fly is self-cured from a trypanosome infection by dissecting after the self-curing process. Therefore it is difficult to relate whether ROS are involved with self-curing (after day 3 post-infection) when dissection takes place 6-8 days after. Hence, an indirect approach was taken to measure ROS levels in tsetse flies exposed to various physiological conditions known to impact susceptibility in flies. Fly age, starvation, fly sex, bloodmeal fractions, immune-stimulation with bacteria, knockdown of immune genes (such as tsetseEP protein) and puparial

conditions were examined to see whether ROS levels in relevant organs in the fly at times known to be critical in determining the outcome of an infection in flies might be induced. In addition, flies were infected with trypanosomes to see whether exposure to trypanosomes themselves causes an induction of ROS.

It has been demonstrated that measurement of hydrogen peroxide (H_2O_2), a free radical, is a good measure of ROS in the insect midgut (Ha *et al.* 2005a). A commercially available kit, Amplex Red (InVitrogen), which uses horseradish peroxidase (HRP) substrate *N*-acetyl-3,7-dihydroxyphenoxazine to catalyze H_2O_2 oxidation to produce a highly red-fluorescent product, resorufin was used (Mohanty *et al.* 1997). Fluorescence can then be measured fluorometrically. This assay was used because of its high sensitivity, increased stability of the oxidized product and its easy reproducibility (Mohanty *et al.* 1997).

This chapter shows that ROS levels change as the teneral fly ages and starved flies have higher ROS levels compared to age-matched fed flies. We finally demonstrate that ROS levels are up regulated when flies are exposed to trypanosomes while bacterial challenges via feeding have no impact on ROS levels in tsetse midguts.

3.2 Materials and methods

3.2.1 Fly maintenance

The colony of tsetse flies was maintained as outlined in section 2.2.1. Groups of experimental flies were collected over a two to four hour time period (depending on number of emerging flies) so that flies were aged 6-10 hours post emergence (p.e) before feeding. For experiments involving flies emerging from puparia reared in different temperatures, puparia were closely monitored every day in order to collect emerging male flies (<6 hrs p.e).

3.2.2 Sample collection

All dissected tissues (midguts and salivary glands) were kept on ice and assayed for H_2O_2 and protein concentrations on the same day. Midguts were dissected into 100 µl of saline on a glass slide, while salivary glands were removed from the fly by slowly teasing them out of the thorax into a 200 µl saline drop.

Fly age assay: Midguts of unfed male flies were dissected 24, 48, 72, 96 and 120 hours post-emergence. The experiment was repeated 3 times; n=75 with n=25/replicate; n=15/timepoint.

Starvation assay: Midguts were dissected from 20 day old male flies that were either fed bloodmeals at normal intervals or fed bloodmeals followed by a period of starvation. The first group consisted of 20 day old flies that were fed normal bloodmeals from day of emergence to dissection (9 normal bloodmeals received in total). The second group consisted of 20 day old flies that were first fed seven bloodmeals at normal intervals and were then starved for the last 7 days of their life. Midguts were dissected from both groups when flies were 20 days old. Three biological replicates were performed; n=35 for fed flies; n=30 for starved flies.

Fly sex assay: Male and female flies were fed normal bloodmeals on the day of emergence. Unfed flies were removed the following day. Midguts and salivary glands were dissected 72 hours post-feeding. Three biological replicates were performed. N=70 for midguts (n=35/sex) and n=60 for salivary glands (n=30/sex).

Blood fractions assay: Male flies were fed either normal horse blood, horse serum only, or heat-inactivated horse serum on day of emergence. Unfed flies were removed the

following day. Midguts were dissected 72 hours post-feeding. Three biological replicates were performed. N=120, with n=40/treatment group.

Immune-stimulation assay: Male flies were fed either live *Escherichia coli* (K12 RM148), dead *E. coli*, dead *Staphylococcus aureus* (SH1000) or LPS-free PBS. Unfed flies were removed the following day. Midguts were dissected 72 hours post-feeding. Three biological replicates were performed. N=120, with n=30/fed live *E. coli*; n=30/ fed dead *E. coli;* n=15/fed dead *S. aureus.*

TsetseEP protein knockdown assay: Male flies were fed normal blood a day after emergence. Fed flies were then injected 24 hours later with either double-stranded (ds) EP or ds enhanced green fluorescent protein (eGFP). Midguts were dissected 72 hours post-dsRNA injection. Three biological replicates were performed. N=30 with n=15 each for dsEP and dseGFP.

Pupal collection assay: Puparia were collected from flies and divided into two treatment groups. Approximately 100 puparia were kept at normal insectary temperature of 26°C (LSTM insectary) and another 100 puparia were incubated at 30°C (Friocell incubator, Amlab, Cambridge, UK). Flies were closely monitored for emerging flies. Female flies emerged first in both groups (approximately 3 weeks for first emerging female flies (30°C) and 4 weeks for first emerging female flies (26°C)). Females were removed and male flies (<6 hours old) emerged 2 to 3 days later. These flies were collected and midguts were subsequently dissected. Three biological replicates were performed. N=30 with n=5/replicate for each temperature.

Trypanosome-infected flies: Male flies were either fed trypanosome-infected blood (*Trypanosoma brucei brucei* strain TSW196 bloodstream form (BSF) parasites) or normal blood on the day of emergence. Unfed flies were removed the following day. Midguts were dissected 72 hours post-feeding. Three biological replicates were performed. N=30 with n=15 per treatment group.

Sodalis glossinidius: *S. glossinidius* were isolated aseptically from tsetse hemolymph and were grown on monolayers of *Aedes albopictus* cells. Twenty days after being in culture, they were adapted to axenic growth and passaged when OD_{600}=0.4. This culture was then gradually adapted to serum-free culture conditions (Haines *et al.* 2002). Clones were isolated onto Mitsuhashi and Maramorosch (MMI) agar plates (1% w/v agar (Difco, Detroit, MI)). Plates were placed in an unvented anaerobic jar (BBL GasPak™) with a BBL CampyPak

Plus™ system (Becton Dickinson, Sparks, MD) to generate microaerophilic conditions. Seven days of incubation at 27°C resulted in white colonies and purity of the culture was monitored by Coomassie Brilliant Blue staining of proteins separated by SDS-PAGE to yield a unique banding pattern (Haines *et al.* 2002) and by PCR using *S. glossinidius* specific primers (O'Neill *et al.* 1993). Isolated cultures were maintained *in vitro* at 27°C in Schneiders medium (PromoCell), SFM (Sigma) and Ex-Cell (SAFC Biosciences). Cultures were centrifuged at 11,500 g for 1 minute in 1.5 ml polypropylene tubes. Supernatants were used to measure ROS levels. Media were used as a control. Three replicates were performed.

3.2.3 Hydrogen Peroxide (H_2O_2) assay to measure ROS levels

An Amplex Red Hydrogen Peroxide Kit from InVitrogen (A22188) was used to measure H_2O_2 levels in dissected tsetse midgut and salivary gland tissues. An initial H_2O_2 standard curve was created by diluting 20 mM H_2O_2 working solution (ranging from 0 µM to 10 µM) into 1X Reaction Buffer (4 ml 5X Reaction Buffer & 16 ml deionized water). A H_2O_2 negative control (1X reaction buffer only) was used to subtract background fluorescence.

Tsetse midguts were dissected into 100 µl saline on a glass slide, while salivary glands were removed by grasping the head and slowing drawing the head, gently teasing the salivary glands out of the thorax into a 200 µl saline drop. The tissues were kept on ice in chilled 1.5 ml polypropylene tubes. Tissues were then homogenized in 25 µl lysis buffer (0.125% Triton 100X, 250 mM Tris HCl, pH 7.4). Tubes were then centrifuged at 12,000 g (Sanyo, UK) for 50 seconds to pellet debris, and 1 µl of the supernatant was pipetted into individual wells of a polystyrene white microplate (Greiner, UK). In order to obtain a final volume of 50 µl solution in each well, 49 µl of 1X reaction buffer was added to each well. Amplex Red reagent/HRP working solution of 50 µl (50 µl 10 mM Amplex Red reagent stock solution, 100 µl 10 U/ml HRP (horseradish peroxidase) stock solution, 4.85 ml 1X reaction buffer) was then added to each microplate well containing standards, controls and tsetse tissue samples. The reaction was incubated at room temperature for 30 minutes and protected from light. Fluorescence emission was detected at approximately 590 nm and an excitation wavelength of 560 nm using a Varioskan machine (Thermo Electron Corporation, UK). To correct for background fluorescence, each fluorescence value obtained had the value from the H_2O_2 negative control subtracted. Three technical replicates of each sample were assayed while all experiments had three biological replicates. H_2O_2 levels were normalized against the protein content of the tissues.

3.2.4 Protein quantification of samples using the Bradford Assay

Bovine serum albumin (BSA) protein standards in lysis buffer ranging from 0.1 to 1.0 mg/ml were prepared in PBS. Each concentration was loaded into a clear Sterilin microplate (5 µl) (Sterilin Limited, UK), and 250 µl of the Bradford Reagent was subsequently added to each well. Supernatants of homogenized tsetse tissues (5 µl) were loaded onto microplates together with 250 µl of the Bradford Reagent and left to incubate at room temperature for 5 minutes. Absorbance was then measured at 595 nm using a VersaMax microplate reader (Molecular Devices, UK). The average from three technical replicates for each sample was the value used for protein concentration. The standard curve was graphed by plotting net absorbance versus protein concentration of each standard.

3.2.5 Serum preparation

Separate blood fractions (blood and serum) were assayed for ROS activity. Defibrinated horse blood (5 ml) (TCS Biosciences Ltd., Buckingham, UK) was centrifuged at 2,000 g on a Jouan benchtop centrifuge for 10 min at 4°C. Serum was then removed and divided into two aliquots. One of the aliquots was heat-inactivated (56°C for 30 min) to remove complement activity. After the serum was heat-inactivated, it was filter sterilized using a 0.2 (µM) micron filter syringe. Flies were then fed either normal blood, normal serum or heat-inactivated serum. Flies were dissected 72 hours post-feeding, the midguts were removed and ROS and Bradford assays were then performed on the fresh tissues.

3.2.6 Bacterial infection

E. coli strain K12 RM148 and *S. aureus* strain SH1000 were grown in LB broth and incubated overnight at 37°C with shaking. Bacterial suspensions were prepared to an OD_{600}=0.5. Heat-killed bacteria were prepared by incubating *E. coli* cells at 75°C for 10 minutes and *S. aureus* cells at 100°C for 15 min. Bacterial death was confirmed by streaking 200 µl of each heat-shocked batch onto LB plates and bacterial growth/death was confirmed after incubation at 37°C 24 and 48 hours after. Bacteria (100 µl) were then diluted in 1 ml defibrinated horse blood and subsequently fed to flies. Unfed flies were removed. Midguts were dissected 72 hours after blood feeding and H_2O_2 and Bradford assays were performed.

3.2.7 Synthesis of double-stranded RNA of tsetseEP protein and eGFP

TsetseEP, a tsetse midgut protein has been shown to be immunoresponsive to both the presence of trypanosomes and Gram-negative bacteria. PCR amplicons tailed with T7 promoter sequences were used to synthesize dsRNAs using the MEGAscript High Yield T7 Transcription kit (Ambion, Huntingdon, UK) according to manufacturer's instructions. TsetseEP DNA plasmid templates were available as clones from the tsetse EST program (Lehane *et al.* 2003). Double stranded eGFP was used as a negative control with the cloning vector pEGFP-N1 (Clontech; GeneBank Accession number U55762) being used as a template for eGFP dsRNA synthesis. Primer sequences are listed below, with T7 promoter sites underlined.

T7EP Forward: 5'- TAATACGACTCACTATAGGGCTACGATAAATATGTCCCTCTAAT – 3'
T7EP Reverse: 5'- TAATACGACTCACTATAGGGTTCTGGCAAACCCTCAAT – 3'
T7eGFP Forward: 5'-TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTTC – 3'
T7eGFP Reverse: 5'- TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGCC – 3'

PCR cycling conditions included: 94°C for 5 minutes, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension of 72°C for 2 min. dsRNA was purified using MEGAclear™ columns (Ambion, Huntingdon, UK), eluted in nuclease free water, and concentrations were determined using a Nanodrop ND-1000 (Wilmington, DE) spectrophotometer. Eluates were further concentrated in a Christ 2-18 rotational vacuum concentrator (Osterode, Germany) to obtain dsRNA concentrations of 3 µg/µl.

3.2.8 Gene knockdown by dsRNA injection

6 µg of dsRNA was injected into each fly as outlined in section 2.2.10.

3.2.9 RNA extraction from collected tissue

To confirm transcript knockdown of tsetseEP protein and the control eGFP, midguts were dissected into PBS, snap frozen in liquid nitrogen and kept at -80°C prior to RNA extraction. RNA extraction was performed as outlined in section 2.2.5.

3.2.10 RT-PCR analysis and assessment of transcript abundance

In order to confirm transcript knockdown of tsetseEP, an RT-PCR reaction was performed using RNA extracted from dissected midguts, with primer sequences listed below. The *G.*

m. morsitans housekeeping gene, GAPDH (Accession number DQ016434) was used as a loading control, with the following forward and reverse primer set:

GAPDH Forward: 5′-CTCAGCTTCTGTGCGTTG-3′
GAPDH Reverse: 5′-AGAGTGCCACCTACGATG-3′
EP Forward: 5′- ACCGTTCGTTCGCTTTACTAC-3′
EP Reverse: 5′- ACCCGCAGCCGTTTGACTTTC-3′

The RT-PCR cycling conditions included: 45°C for 45 minutes, 95°C for 2 min, 30 cycles of 95°C for 45 seconds, 54°C for 1 min, 68°C for 1 min and a final extension of 68°C for 5 min. RT-PCR products were analyzed by gel electrophoresis using 1.5% agarose gel. Band intensities were measured using Gene Tools software on a Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

3.2.11 Trypanosome infections

Flies were infected as outlined in section 2.2.2. Flies were fed the infectious blood at the first bloodmeal and unfed flies were removed the following day. Midguts of flies were dissected 72 hours post-infection, shredded into 100 µl of saline on a glass slide and infection status was scored by searching for motile trypanosomes in 10 random fields using dark field microscopy (125 X magnification). Tissues were collected into 1.5 ml chilled polypropylene tubes and kept on ice until the subsequent assays for H_2O_2 concentrations and total protein content.

3.2.12 Statistical analysis

Statistical analysis was performed using SPSS16 (SPSS Inc., Chicago, Illinois). Student's t-test was performed to determine if significant differences were present between two groups and ANOVA was performed for multiple analysis. Differences were deemed significant if a p-value was less than 0.05. A bivariate 2-tailed correlation analysis (ANOVA) was performed on fly age and midgut infection prevalence to check for correlation. Correlation was significant for p-values less than 0.05.

3.3 Results

3.3.1 Baseline H_2O_2 measurements in tsetse midguts and salivary glands

H_2O_2 measurements from tsetse midguts and salivary glands were pooled together to obtain baseline measurements from flies that were of similar ages (Figure 3.1). H_2O_2 concentrations from the midguts of age-matched male and female flies were assayed. Baseline H_2O_2 concentrations from midguts of flies dissected in April were higher (1.8 ± 0.72 µM per mg/ml) than baseline concentrations from September (1.29 ± 0.15 µM per mg/ml) although differences were not statistically different (p-value = 0.19). H_2O_2 measurements in salivary glands were also not statistically different (p-value = 0.565) between males and females. Large error bars are due to variation between experiments since the H_2O_2 measurements are accumulated from data generated from different experiments. Data for H_2O_2 measurements in salivary glands is from one experiment (three replicates). Due to no significant differences between H_2O_2 measurements in midguts and salivary glands, the latter was not pursued further throughout the chapter.

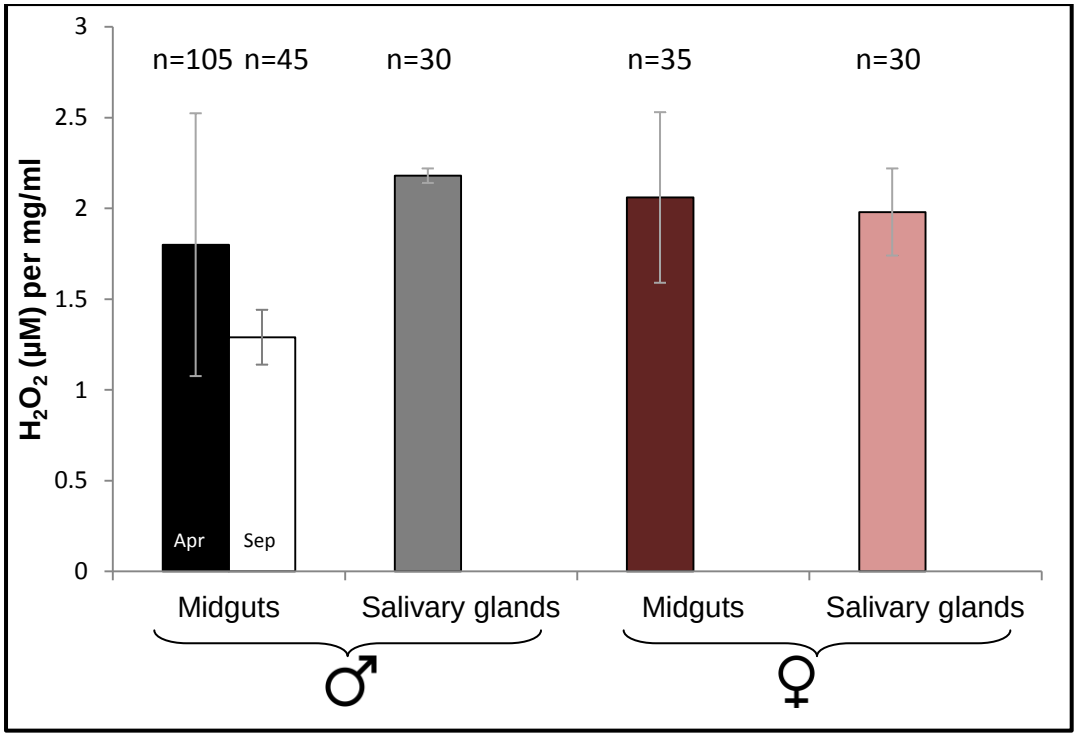


Figure 3.1. Average H_2O_2 concentrations (µM) per mg/ml isolated from pooled whole midguts and paired salivary glands of age-matched male and female flies. Baseline midgut H_2O_2 concentrations were obtained from pooled measurements obtained from data pooled from experiments performed in April (Apr; black bar; three experiments/nine replicates) and September (Sep; white bar; three experiments/nine replicates). The H_2O_2 concentrations of female midguts and salivary glands (one experiment; three replicates). Standard error is represented by the error bars for nine biological replicates for male midguts while standard error for the remaining treatment groups are represented by error bars for three biological replicates. N=number of flies/group. H_2O_2 levels are represented as concentration (µM) of H_20_2 per mg/ml of protein in the reaction mixture but can be converted to enzyme reaction kinetic values (pmoles H_2O_2 converted per min per mg/ml protein) by multiplying value by 25 µl (total volume of extract) X 100 (1 µM = 100 pmoles per 100 µl reaction volume) and dividing by 30 (time of incubation); ie. a factor of 83.33.

3.3.2 ROS levels change as a teneral fly ages

The teneral phenomenon refers to the susceptibility to parasite infection of newly emerged flies that have been infected with trypanosomes in the first bloodmeal. This is a phenomenon unique to tsetse. It is widely observed that newly emerged flies are more susceptible to infection than flies that have had previous bloodmeals (Haines *et al.* 2010; Walshe *et al.* 2011). If ROS are involved in establishment of trypanosomes in the midgut it might be expected that there would be a correlation between ROS and prevalence. The midguts from unfed male flies were dissected over a timecourse of 120 hours post-emergence (every 24 hours). The quantity of H_2O_2 was measured in these tissues.

Figure 3.2 shows that ROS levels are influenced by fly age. Young flies have basal ROS levels (24 hours: 2.44 ± 0.16 µM per mg/ml), however as flies begin to mature, ROS levels increase (48 hours: 2.13 ± 0.18 µM per mg/ml, 72 hours: 2.85 ± 1.05 µM per mg/ml and 96 hours: 3.57 ± 0.96 µM per mg/ml). However flies between 48 and 96 hours of emergence are ready to feed; by 96 hours post-emergence, unfed flies have likely entered a starvation state and by 120 hours post-emergence, starvation reaches critical state (observed increased mortality of approximately 67%; open grey diamonds in Figure 3.2). Small error bars are seen in ROS levels from 24 and 48 hours post-emergence compared to rather large error bars in ROS levels from 72, 96 and 120 hours post-emergence. The small error bars could possibly be due to starvation state not being very critical at these time points (24 and 48 hours), and therefore H_2O_2 values do not vary in these flies. The large error bars could be due to starvation (72, 96 and 120 hours), confirmed by increased mortality. Starvation states vary depending on each fly, particularly at these time points, therefore fluctuations in H_2O_2 levels are observed in these flies, hence large error bars.

Recently, it was shown that post-eclosion age predicts the prevalence of midgut trypanosome infections in tsetse (Walshe *et al.* 2011). A linear relationship has been shown between infection prevalence up to 72 hours-post emergence, using the same batch of flies and *T. b. brucei* TSW196 BSF stabiliates. Therefore, expected midgut infection prevalence was calculated using the linear regression analysis giving y=-0.68x + 79.20 up to 72 hours post-emergence (Walshe *et al.* 2011). Table 3.1 shows the expected midgut infection prevalence from 24 to 120 hours post-emergence and the H_2O_2 levels assayed at those time points.

A bivariate correlation coefficient for the entire experiment (24 – 120 hours) between ROS levels and midgut infection prevalence was 0.671 and was therefore not significant

(correlation is significant at the 0.05 level). Therefore, although ROS levels change as a teneral fly ages, from 24 to 120 hours, no correlation exists between ROS levels and prevalence of midgut infections.

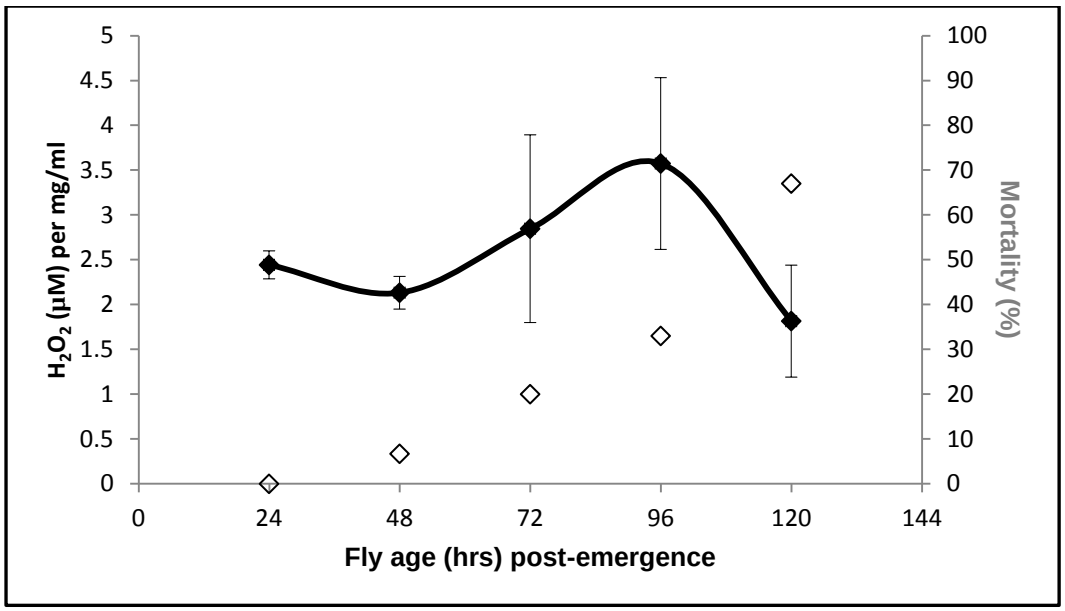


Figure 3.2. Differential expression of H_2O_2 measurements in teneral flies upon aging and mortality (%). Timecourse of midgut H_2O_2 concentrations (µM) per mg/ml plotted on y-axis (left) of male teneral flies aged 24, 48, 72, 96 and 120 hrs post-emergence. Mean ± SE for three biological replicates (n=75). Mortality (%) is shown in open grey diamonds plotted on y-axis (right). H_2O_2 levels are represented as concentration (µM) of H_20_2 per mg/ml of protein in the reaction mixture but can be converted to enzyme reaction kinetic values (pmoles H_2O_2 converted per min per mg/ml protein) by multiplying value by 25 µl (total volume of extract) X 100 (1 µM = 100 pmoles per 100 µl reaction volume) and dividing by 30 (time of incubation); ie. a factor of 83.33.

Table 3.1. H_2O_2 measurements extracted from graph above for male teneral flies 24 to 120 hrs post-emergence and typical midgut parasite prevalence (%) observed under laboratory conditions. H_2O_2 measurements were from midguts sampled every 24 hours post-emergence ± standard error of the mean (S.E.M). H_2O_2 levels are represented as concentration (µM) of H_20_2 per mg/ml of protein in the reaction mixture but can be converted to enzyme reaction kinetic values (pmoles H_2O_2 converted per min per mg/ml protein) by multiplying value by 25 µl (total volume of extract) X 100 (1 µM = 100 pmoles per 100 µl reaction volume) and dividing by 30 (time of incubation); ie. a factor of 83.33.Expected/calculated parasite midgut prevalence (%) in *G. m. morsitans* when fed *T. b. brucei* TSW196 BSF under laboratory conditions obtained from reported experiments carried out in the laboratory (Walshe *et al.* 2011). Bivariate correlation coefficient between 24 – 120 hours = 0.671 (not significant).

	24 hrs	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**
H_2O_2 (µM) per mg/ml ± S.E.M	2.44 ± 0.16	2.13 ± 0.18	2.85 ± 1.05	3.57 ± 0.96	1.82 ± 0.62
% midgut prevalence	62.88	46.56	30.24	~<10%	~<10%

3.3.3 Starved flies have lower ROS levels compared to age-matched fed flies

It is known that susceptibility of older flies to trypanosome infection can be significantly increased by a period of starvation prior to ingestion of an infected bloodmeal (Gingrich *et al.,* 1982; Kubi *et al.,* 2006). This provides another opportunity to test the hypothesis that ROS has a direct relationship to the ability of trypanosomes to establish in the fly midgut by determining if nutritionally induced changes in ROS levels can be correlated with susceptibility of the fly to trypanosomes. H_2O_2 levels of 20 day old male flies fed normal bloodmeals (9 bloodmeals received in total) were compared with age-matched flies that were 'starved' for one week after the seventh bloodmeal. Figure 3.3 Panel A shows that ROS levels in fed flies (1.72 ± 0.14 µM per mg/ml) are significantly lower (1.4 fold, p-value = 0.016) than starved flies (2.35 ± 0.21 µM per mg/ml).

In our attempt to correlate parasite susceptibility of fed and starved (non-teneral) flies to ROS levels, midgut infection prevalence (%) data was obtained from literature from flies that were matched by age to our ROS assayed flies. Twenty day old male flies that were infected on the tenth bloodmeal would typically have low midgut infection rates of less than 5% (Haines *et al.* 2010). In contrast, 20 day old flies that have received at least one bloodmeal prior to the infective bloodmeal followed by a severe starvation period (7 days) resulted in a higher midgut infection prevalence (25% *T. b. brucei* and 49% *T. congolense*) compared to the non-starved flies (2.9% *T. b. brucei* and 3.9% *T. congolense*) (Kubi *et al.* 2006). Our flies assayed for H_2O_2 concentrations were also 20 days old and endured 7 days of starvation prior to dissection. The midgut infection prevalence between fed and starved flies was reported to be an 8.6 fold increase in starved *T. b. brucei*-infected flies and 12.6 fold increase in starved *T. congolense*-infected flies (Figure 3.3 Panel B). This evidence suggests that increased susceptibility of flies following starvation is not explained by lowered ROS defences. However, the synergistic effect of ROS and NO to produce peroxynitrite cannot be ruled out.

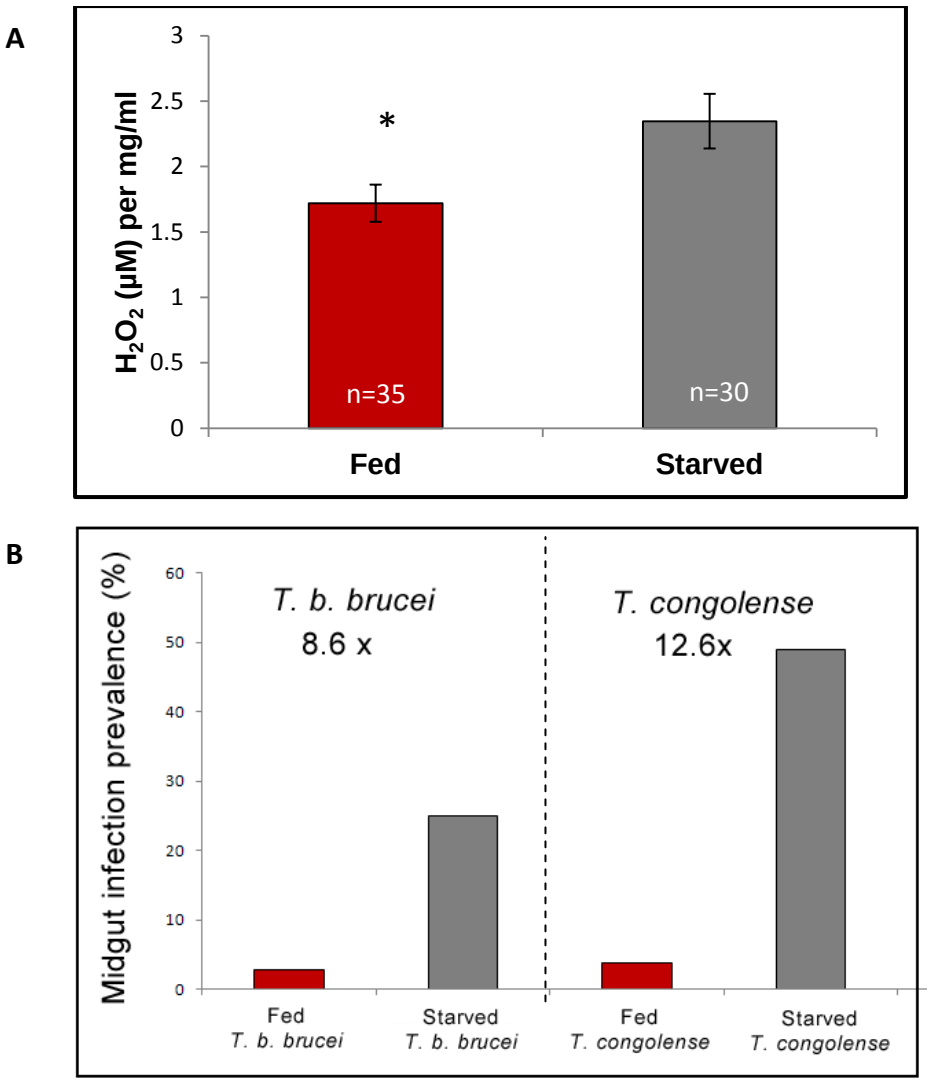


Figure 3.3. H_2O_2 measurements in midguts from 20 days old starved flies compared to age-matched unstarved flies and the approximate midgut infection prevalence in these flies. (A) H_2O_2 concentrations (µM) per mg/ml for age-matched (grey) starved flies (7 bloodmeals received prior to initiating a 7 day starvation period) and fed (red) flies (9 bloodmeals received). Mean ± SE for three biological replicates; * =p-value of 0.016. **(B)** Midgut infection prevalence (%) in fed flies given previous bloodmeals prior to the infective parasitic bloodmeal at (10^{th} bloodmeal) (red) and in starved flies that received at least one bloodmeal prior to the infective bloodmeal in 20 day old adults starved for 7 days (grey). Flies were infected with either *T. b. brucei* or *T. congolense.* Data on midgut infection prevalence (%) was obtained from previous data (Kubi *et al.* 2006; Haines *et al.* 2010). Differences in midgut infection prevalence are indicated by fold differences (8.6 x and 12.6 x) for starved *T. b. brucei* and *T. congolense* infected flies compared to fed flies. H_2O_2 levels are represented as concentration (µM) of H_20_2 per mg/ml of protein in the reaction mixture but can be converted to enzyme reaction kinetic values (pmoles H_2O_2 converted per min per mg/ml protein) by multiplying value by 25 µl (total volume of extract) X 100 (1 µM = 100 pmoles per 100 µl reaction volume) and dividing by 30 (time of incubation); ie. a factor of 83.33.

3.3.4 Levels of ROS between tsetse sexes do not differ significantly

Evidence suggests that male flies mature more trypanosome infections than female flies (Milligan *et al.* 1995). However, female flies from our *G. m. morsitans* colony are more susceptible to *T. b. brucei* and *T. congolense* infections than male flies (Walshe *et al.* 2011). This presents another opportunity to test the hypothesis that ROS has a direct link in the ability of either sex to mature more trypanosome infections if differing H_2O_2 levels in both sexes can be correlated with susceptibility to trypanosome infections. Since maturation of trypanosome infections depends on multiple points (midgut proventriculus and salivary glands) during the development cycle, H_2O_2 levels were investigated in the midguts (including proventriculus) and salivary glands of fed male and female flies 72 hours post-feeding. Figure 3.4 Panel A shows no significant differences between H_2O_2 levels in midguts (p-value = 0.431) or salivary glands (p-value = 0.565) of male and female tsetse flies. This suggests that the sex-dependent susceptibility to trypanosome infection is not due to ROS.

Although flies were collected over a 24 hour period to minimize age-related discrepancies, the standard errors between midguts and salivary glands differ; H_2O_2 measurements of midguts have small standard errors (male ± 0.08 µM per mg/ml and female ± 0.04 µM per mg/ml), indicating that there is high reproducibility between replicates where the mean ± S.E is from three replicated experiments. The H_2O_2 levels in salivary glands fluctuated greatly, which caused large standard errors in both males (± 0.47 µM per mg/ml) and females (± 0.24 µM per mg/ml). The physiological basis for these differences is unknown.

3.3.5 ROS levels are not influenced by different fractions of a bloodmeal

It has been reported that removing serum from the bloodmeal increases parasite infection rates in male tsetse (Maudlin *et al.,* 1984). This presents another opportunity to test the hypothesis that ROS influence trypanosome establishment in tsetse midgut by determining if changes in H_2O_2 levels induced by different fractions of a bloodmeal can be correlated with fly susceptibility to trypanosome infections. To measure H_2O_2 levels in midguts of flies fed different fractions of a bloodmeal, flies were fed normal blood, serum or heat-inactivated serum in the first bloodmeal. Midguts were dissected 72 hours post-feeding to reduce excess blood contamination in the midgut tissues. Figure 3.4 Panel B shows that ROS expression did not change when flies were fed on the different fractions of blood. Differences between the three groups were not significant. Flies that fed solely on serum had H_2O_2 concentrations of 1.89 ± 0.17 µM per mg/ml, heat-inactivated serum (1.92 ± 0.16 µM per mg/ml) and blood (1.82 ± 0.18 µM per mg/ml). This data also suggests that flies

fed on serum (1.7 µM H_2O_2/fly), heat-inactivated serum (1.7 µM H_2O_2/fly) or normal blood (1.63 µM H_2O_2/fly) are consistent with basal amounts of H_2O_2 in male flies that are in a steady state. This was calculated to be approximately 1.6 µM H_2O_2/fly fed on normal blood (approximately 1.8 µM per mg/ml of midgut protein) based on previous experiments, although calculations of H_2O_2 per fly were approximate since each fly ingested different volumes of blood/serum. This suggests that susceptibility to trypanosome infections when flies are fed different fractions of a bloodmeal cannot be explained by ROS levels.

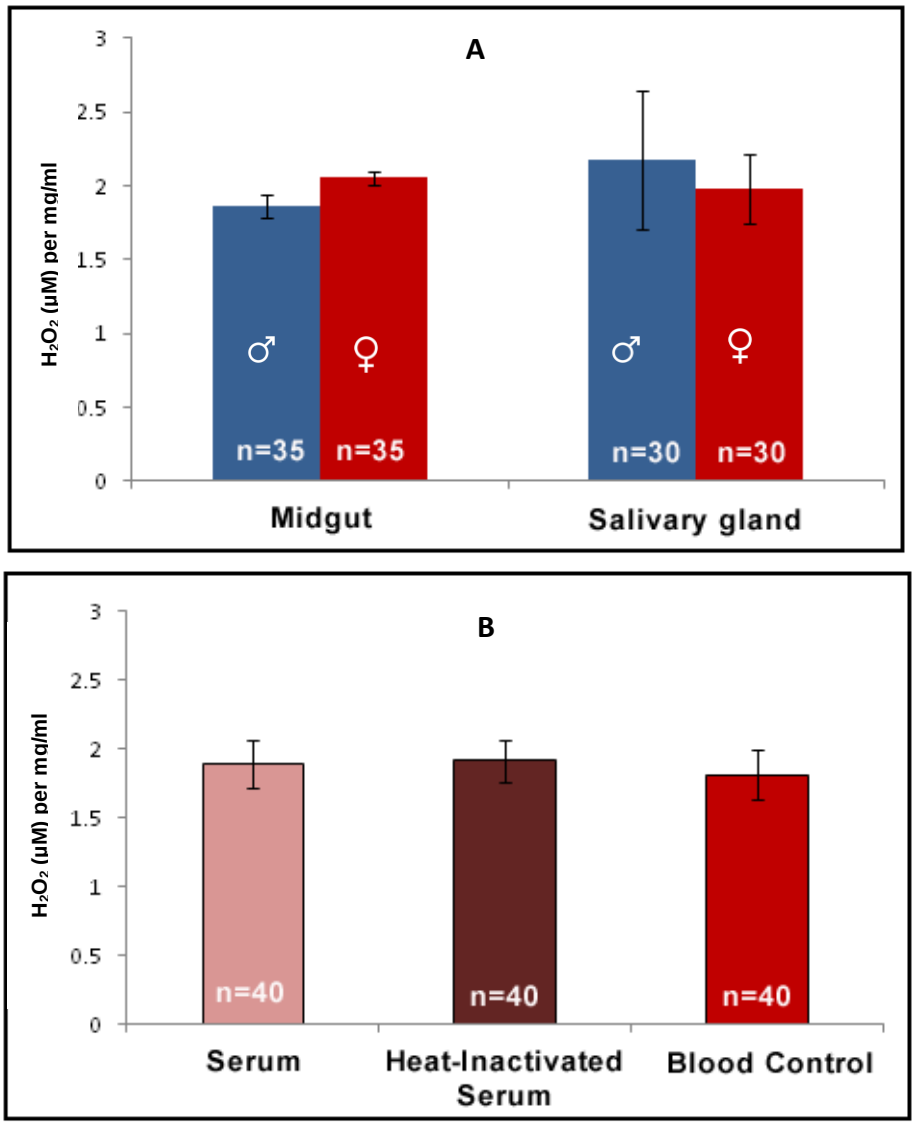


Figure 3.4. H_2O_2 measurements in midguts and salivary glands isolated from age-matched male and female tsetse flies and H_2O_2 measurements in midguts of flies fed on serum, heat-inactivated serum or normal blood. (A) H_2O_2 concentrations (µM) per mg/ml for midgut and salivary glands of male and female flies. Mean ± SE for three biological replicates. N=total number of flies dissected. **(B)** H_2O_2 concentrations (µM) per mg/ml in fly midguts dissected 72 hours post-feeding. Mean ± SE for three biological replicates; n=flies/treatment group. H_2O_2 levels are represented as concentration (µM) of H_20_2 per mg/ml of protein in the reaction mixture but can be converted to enzyme reaction kinetic values (pmoles H_2O_2 converted per min per mg/ml protein) by multiplying value by 25 µl (total volume of extract) X 100 (1 µM = 100 pmoles per 100 µl reaction volume) and dividing by 30 (time of incubation); ie. a factor of 83.33.

3.3.6 Immune stimulation of flies with bacteria does not result in higher ROS levels

It has been shown that tsetse receiving an immune-stimulus prior to receiving a parasite-infected bloodmeal develop significantly fewer midgut infections (Hao *et al.* 2001). Although this has been linked to antimicrobial peptide induction, other changes in the fly upon bacterial challenge cannot be discounted (Hao *et al.* 2001; Hao *et al.* 2003). Immune challenge-induced changes in systemic levels of free radicals might make the midgut environment hostile for trypanosome development. Therefore, whether H_2O_2 levels are induced in tsetse midguts after bacterial challenge were tested. Flies were fed live *E. coli,* dead *E. coli* or LPS-free PBS, and the midguts were dissected 72 hours after the spiked bloodmeal.

Figure 3.5 Panel A shows that ROS levels between the 3 treatment groups, live *E. coli* (1.95 ± 0.31 µM per mg/ml), dead *E. coli* (1.85 ± 0.32 µM per mg/ml) and LPS-free PBS (1.86 ± 0.32 µM per mg/ml) were not significantly different (p-value between PBS control and dead *E. coli* = 0.128; p-value between PBS control and live *E. coli* = 0.573). H_2O_2 concentrations in these flies remained consistent with baseline H_2O_2 concentration in the midgut (1.8 µM per mg/ml) although we observed increased mortality rates in flies fed live *E.coli* spiked bloodmeal. Since no changes in H_2O_2 expression were detected with Gram-negative bacteria, we decided to feed flies with Gram-positive bacteria instead to check whether H_2O_2 expression might be dependent on the type of bacteria. Therefore, the same experiment was repeated using dead *S. aureus* and LPS-free PBS, and the results indicate that H_2O_2 production does not increase upon Gram-positive bacterial stimulation as levels of H_2O_2 were again similar to baseline concentration (1.25 µM per mg/ml) (Figure 3.5 Panel B). Differences in H_2O_2 measurements between the PBS control (1.16 ± 0.17 µM per mg/ml) and *S. aureus*-fed flies (1.44 ± 0.48 µM per/mg) were not significant (p-value = 0.703). A noticeable drop (approximately 60%) in H_2O_2 measurements between PBS control groups in *E. coli* experiment and *S. aureus* experiment was observed. Differences in sample sizes between the two experiments (n=30 for *E. coli* and n=15 for *S. aureus;* Figure 3.5 Panel A and B) were due to the availability of age-matched teneral flies that were less than 24 hours old. Consequently, bacterial challenges do not induce a ROS response in tsetse midguts. In addition, the increased refractoriness of flies following immune stimulation is not due to H_2O_2.

3.3.7 *Sodalis glossinidius* does not induce ROS levels

H_2O_2 levels in the supernatant of *in vitro* cultured *S. glossinidius* (the secondary tsetse symbiont) were measured to determine whether the Gram-negative endosymbiont independently generates ROS. *Sodalis* is believed to increase trypanosome midgut infection in the initial bloodmeal in tsetse (Maudlin and Ellis 1985; Maudlin *et al.* 1990), therefore if the hypothesis is correct then we would expect low ROS levels in *Sodalis* cultures if ROS are correlated with high susceptibility to trypanosome infections. However, we are aware of the narrow discerning power of this experiment compared to *in vivo* work. *Sodalis* culture has slightly reduced H_2O_2 levels (4.76 ± 0.26 µM per mg/ml) compared to its medium control (5.55 ± 0.06 µM per mg/ml), although the differences are not significant (p-value = 0.067) (Figure 3.5 Panel C). Consequently, *Sodalis* does not produce detectable H_2O_2.

3.3.8 Trypanosomes present in the bloodmeal can induce ROS expression

Tsetse were infected with bloodstream form (BSF) trypanosomes at the first bloodmeal and H_2O_2 levels were assayed to see if ROS are induced upon parasite infection. Seventy-two hours after infection all flies that fed on an infected bloodmeal were infected with trypanosomes therefore, we could not differentiate between an infected fly and a fly that would eventually become self-cured. All infected flies had active trypanosomes in their midguts. ROS levels were significantly increased (p-value = 0.029) in trypanosome-infected flies (2.08 ± 0.11 µM per mg/ml) compared to non-infected flies (1.44 ± 0.01 µM per mg/ml) (Figure 3.5 Panel D) suggesting that trypanosome infection induces an immune defence involving H_2O_2.

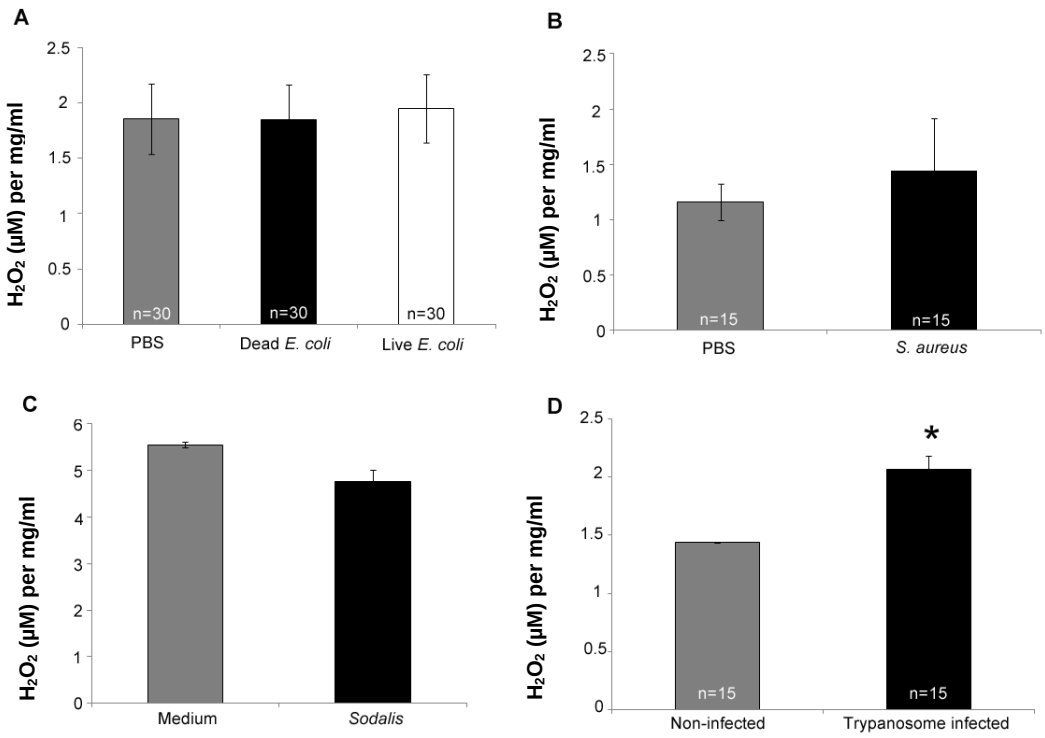


Figure 3.5. H_2O_2 measurements in flies fed with LPS-free PBS, Gram-negative bacteria *(E. coli)* or Gram-positive bacteria *(S. aureus)*; H_2O_2 concentrations of *in vitro* cultured *Sodalis glossinidius* and naive-medium and H_2O_2 concentrations from midguts of flies 72 hours post-feeding on either parasite-free (naive) blood or infected blood (trypanosome infected). (A) Flies were fed either LPS-free PBS, dead *E.coli* or live *E.coli*; n=number of flies/replicate. Midguts were dissected 72 hours post-feeding. **(B)** Flies were fed either LPS-free PBS or dead *S. aureus;* n=number of flies/replicate. Midguts were dissected 72 hours post-feeding. **(C)** *S. glossinidius* cultures were assayed for ROS levels. The medium was measured as a control for baseline levels. **(D)** Flies were infected with TSW196 BSFs or fed uninfected normal blood. N=number of flies/group; *=p-value of 0.029. Mean ± SE for three biological replicates. H_2O_2 levels are represented as concentration (µM) of H_20_2 per mg/ml of protein in the reaction mixture but can be converted to enzyme reaction kinetic values (pmoles H_2O_2 converted per min per mg/ml protein) by multiplying value by 25 µl (total volume of extract) X 100 (1 µM = 100 pmoles per 100 µl reaction volume) and dividing by 30 (time of incubation); ie. a factor of 83.33.

3.3.9 Gene knockdown of tsetseEP protein does not alter levels of ROS

Haines *et al.* (2005) have shown that knockdown of tsetseEP protein results in increased parasite prevalence in tsetse midgut. The mechanism by which tsetseEP protein influences parasite survival in the midgut is unknown. Therefore, other than tsetseEP protein having a direct impact on parasites, we wanted to determine whether ROS levels might be subjective to tsetseEP protein concentration in the midgut. If ROS contributed to this phenotype; then, according to our hypothesis, we could expect a reduction in ROS levels when tsetseEP protein is knocked down. Figure 3.6 Panel A shows that flies injected with dsEP (1.14 ± 0.22 µM per mg/ml) and dseGFP (1.28 ± 0.20 µM per mg/ml) have similar ROS levels (p-value = 0.164) in the midguts of these flies. This suggests that ROS levels are not influenced by levels of tsetseEP protein, and that the phenotype of increased midgut parasite prevalence upon knockdown (confirmed by RT-PCR at 70.88% knockdown; insert in Figure 3.6 Panel A) is not associated with ROS levels. Although differences were not significant, H_2O_2 concentrations in flies injected with dsEP are slightly lower than dseGFP control injected flies. The latter had almost similar baseline H_2O_2 levels observed in a steady state fly (1.29 µM per mg/ml for experiments carried out in September 2011). Although no western blots were performed to confirm protein knockdown, Haines *et al.* (2010) have shown that transcript knockdown typically results in complete elimination of the endogenous tsetseEP protein (Haines *et al.* 2010).

3.3.10 Midgut ROS levels are not influenced by the temperature at which the puparia were exposed during development

It has been reported that puparia of *G. m. morsitans* that receive a period of elevated temperature during pupation, produce more parasite-susceptible flies (Burtt 1946). If according to our hypothesis, ROS are involved in this temperature-induced susceptibility, then ROS levels should vary between heat shocked puparia and puparia reared under normal conditions. Midgut ROS levels from mature teneral flies emerging from puparia continually exposed to a temperature of 26°C (insectary) or 30°C (incubator) were compared. Humidity in the 30°C incubator was lower (average 64% relative humidity) than that in the insectary which was on average 71% relative humidity. Appendix I shows the temperature and humidity chart from insectary (26°C) where the puparia were kept.

Figure 3.6 Panel B shows no significant differences (p-value = 0.396) in ROS levels between flies that have emerged from puparia reared at 26°C (3.67 ± 0.87 µM per mg/ml) and

puparia reared at 30°C (4.52 ± 0.61 µM per mg/ml). However, it is interesting to note that levels of H_2O_2 are almost 3.5 times higher in both groups than baseline levels of flies from same time period (1.29 µM per mg/ml in Figure 3.1). There were no visual physiological differences between puparia reared at these two temperatures and no physical differences between flies emerging between the two treatment groups. After newly emerged flies were dissected for midgut tissues for the ROS assay, remaining puparia were discarded, therefore, mortality rates of emerging flies were not monitored, particularly flies emerging from puparia kept at 30°C. In both cases, female flies emerged first, with male flies emerging 2-3 days after. Flies emerged approximately 1 week earlier from puparia kept at 30°C than puparia kept at 26°C. Thus, increased susceptibility of flies emerging from heat shocked puparia is not explained by ROS levels.

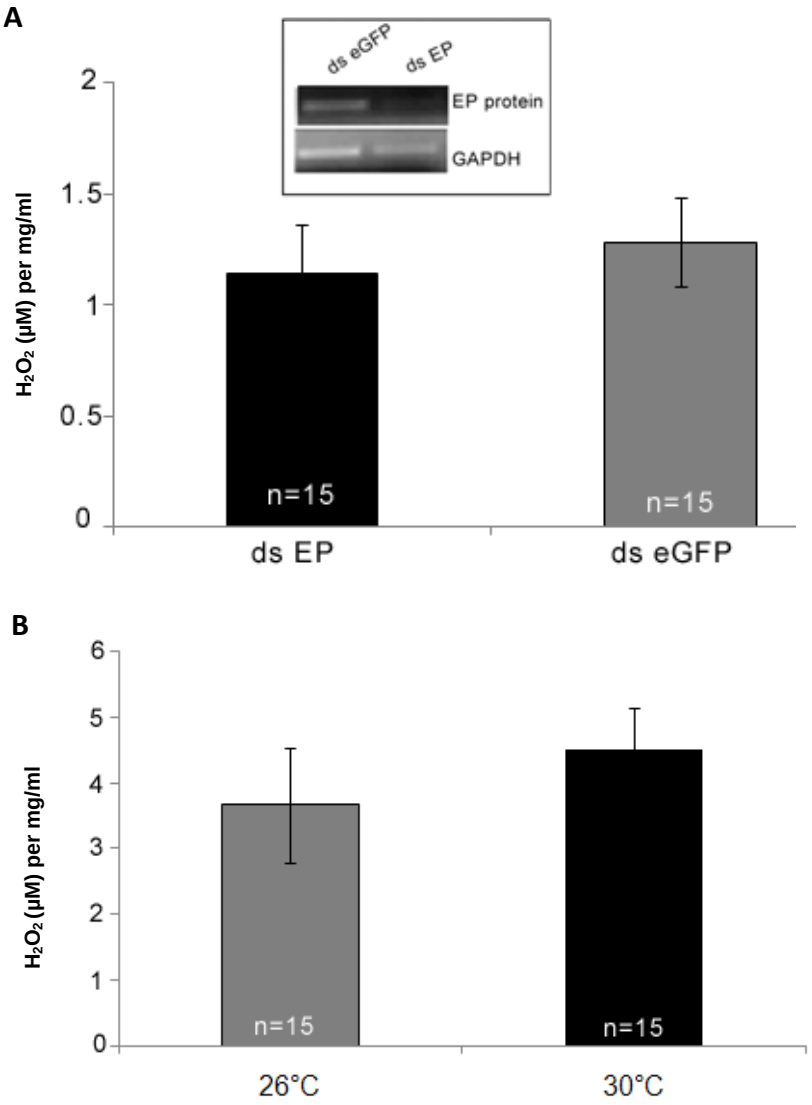


Figure 3.6. H_2O_2 concentrations (µM) per mg/ml in midguts of flies dissected 72 hours post dsEP or dseGFP injection and H_2O_2 concentrations in midguts of flies emerging from puparia kept at either 26°C or 30°C. (A) Mean ± SE for three biological replicates. N = number of flies/group. Insert: 72 hours post-dsRNA injection, transcript levels of tsetse EP protein were significantly reduced in midgut as determined by semi-quantitative RT-PCR with housekeeping gene, GAPDH as a control. **(B)** Standard error is represented by the error bars for three biological replicates. N=number of flies/group. H_2O_2 levels are represented as concentration (µM) of H_20_2 per mg/ml of protein in the reaction mixture but can be converted to enzyme reaction kinetic values (pmoles H_2O_2 converted per min per mg/ml protein) by multiplying value by 25 µl (total volume of extract) X 100 (1 µM = 100 pmoles per 100 µl reaction volume) and dividing by 30 (time of incubation); ie. a factor of 83.33.

3.4 Discussion

In *Glossina,* increased susceptibility to trypanosome infections are seen when the first bloodmeal taken is infected with trypanosomes. Higher trypanosome susceptibility is observed in newly emerged, unfed flies compared to older, previously fed flies. This phenomenon in tsetse is known as the teneral phenomenon. Typically in the laboratory, infections with bloodstream forms of *T. congolense* and *T. b. brucei* decreases from maximal values of 70% to 10% and less after the third bloodmeal (Distelmans *et al.* 1982; Welburn and Maudlin 1992). In the field, tsetse can become infected with *T. congolense* infections at any age although a decrease in susceptibility is seen with fly age, due to either the age of the fly or increased numbers of refractory flies (Msangi *et al.* 1998; Woolhouse and Hargrove 1998; Kubi *et al.* 2007). In the field, the teneral phenomenon may not be as obvious since the situation with *T. brucei* infections are unknown due to fly infections being low in the field (Walshe *et al.* 2011).

If ROS were involved with this susceptibility phenotype, then we could predict that high susceptibility would equate with low midgut ROS levels. In procyclics forms (PCFs) and bloodstream forms (BSFs) of *T. brucei,* ROS have been shown to act as intermediaries in the signalling pathway leading to programmed cell death (PCD) (Ridgley *et al.* 1999; Fang and Beattie 2003; Duszenko *et al.* 2006; Figarella *et al.* 2006; Goldshmidt and Michaeli 2011). *T. b. brucei* PCFs incubated with xanthine oxidase (enzyme involved in ROS synthesis) activates a Ca^{2+}-dependent pathway resulting in cell death of the parasites (Ridgley *et al.* 1999; Wang *et al.* 2002). Furthermore, incubation with ROS scavengers *N*-acetyl-L-cysteine (NAC) and glutathione (GSH) hinders ROS production and PCD in *T. brucei* BSFs (Figarella *et al.* 2006). Furthermore, overexpression of trypanosome alternative oxidase (TAO) in *T. brucei* PCFs leads to reduced ROS production and prevents PCD-like phenomena in *T. brucei* BSFs (Fang and Beattie 2003; Tsuda *et al.* 2005).

H_2O_2 levels in tissues of tsetse exposed to various physiological conditions known to impact susceptibility to trypanosomes were measured. Overall our data suggests that ROS do not play a central role in determining susceptibility of tsetse flies to trypanosomes. No correlation between ROS levels and prevalence of midgut trypanosome infections in tsetse from 24 to 120 hours post-emergence is observed. The general observed trend in ROS levels in aging flies is possibly due to starvation. Periods of extreme starvation (seven days

for an adult fly and three to four days for a teneral fly) suppress the physiological factors that constitute a developmental barrier for a trypanosome infection (Kubi *et al.* 2006).

A separate experiment was carried out to examine whether starvation-induced susceptibility could be due to a change in midgut ROS levels. Adult tsetse flies, although typically refractory to parasite establishment, can still acquire mature trypanosome infections and a starvation event has been shown to increase the flies' susceptibility to these trypanosomes (Gingrich *et al.* 1982b). The nutritional status of tsetse at the time of imbibing the infective bloodmeal can also affect whether tsetse flies are able to successfully vector both *T. congolense* and *T. b. brucei* parasites (Kubi *et al.* 2006). Lower H_2O_2 levels in fed flies (more refractory to trypanosome infection) were recorded than starved flies (more susceptible to trypanosome infection). Thus, parasite susceptibility in starved flies is not likely linked to decreased ROS levels, but perhaps instead due to the compromised nutritional state of flies. Starvation depletes energy reserves, which could lead to a distressed immune response. This would create a less hostile midgut environment for the ingested trypanosomes and thus favour establishment.

The unexpected decrease in H_2O_2 levels in fed flies could also be associated with the release of haem via bloodmeal-associated haemoglobin degradation. Haemoglobin, the main blood protein, when digested by the insect releases large amounts of haem (Oliveira *et al.* 2011). Unbound haem is a potent pro-oxidant that induces oxidative stress by generating reactive oxygen species that can damage DNA, proteins and membrane lipids (Oliveira *et al.* 2011; Tripodi *et al.* 2011). Therefore, protective mechanisms are in place to compensate for the damaging toxic effects of haem and ROS. Detoxification mechanisms in various organisms include haem degradation (Lara *et al.* 2005; Paiva-Silva *et al.* 2006), haem aggregation (Oliveira *et al.* 1999; Oliveira *et al.* 2007), expression of antioxidant enzymes (Paes *et al.* 2001; Diaz-Albiter *et al.* 2011) and haem-binding proteins (Oliveira *et al.* 1995; Paiva-Silva *et al.* 2002). Therefore, detoxifying mechanisms decrease ROS levels and help compensate for the ingestion of haem (Molina-Cruz *et al.* 2008; Oliveira *et al.* 2011). Levels of detoxifying enzymes should be measured by using commercially available kits such as the Amplex Red Catalase Assay Kit (InVitrogen) to further validate their induction. These results suggest that other factors influence the increased susceptibility of starved flies to trypanosome infection.

It has been previously reported that removal of serum from the bloodmeal leads to an increase in midgut infection rates (Maudlin *et al.,* 1984). Trypanosomes (midgut procyclics)

are highly susceptible to vertebrate host serum and this susceptibility has been linked to serum complement activity (Ferrante 1983; Black *et al.* 1999; Ooi 2011). The complement cascade (made up of serum proteins, glycoproteins and receptors) is part of innate host immunity to help fight off infection (Ferrante 1983). The complement cascade complements the adaptive immune response through antigen-antibody interactions and the humoral response against specific antigens (Mastellos *et al.* 2003). Therefore, the different fractions that constitute a bloodmeal were tested to see if the trypanolytic activity, particularly in serum, could also be due to ROS rather than complement alone. Feeding of serum removes complications associated with haem, which is only found in red blood cells. Flies were fed normal blood, serum or heat-inactivated serum to inactivate complement proteins since it has been shown that the trypanolytic activity in bovine serum can be inactivated by heating the serum at 56°C for 30 min (Ferrante 1983). The blood fractions fed to tsetse were found to have no influence on midgut ROS levels. This is not surprising since a wild tsetse feeds on multiple hosts (Leak 1999). If different bloodmeal fractions played a role in ROS, then H_2O_2 levels would constantly be fluctuating, leading to unhealthy oxidative stress to the fly. H_2O_2 expression does not change when tsetse feed on normal blood, serum or heat-inactivated serum.

To investigate if fly sex has an effect on ROS levels in tsetse, we measured H_2O_2 levels in midguts and salivary glands of both male and female tsetse flies. Previous evidence suggests that male flies contract more trypanosome infections than female flies (Otieno *et al.* 1983; Milligan *et al.* 1995). However some studies have reported increased female susceptibility to parasite infection (Mwangelwa *et al.* 1987; Aksoy *et al.* 2003; Walshe *et al.* 2011). In addition, the sex-based bias in parasite susceptibility varies with different vector-parasite combinations. Teneral female *G. m. morsitans* were more susceptible to *T. b. brucei* infections while teneral male *G. p. palpalis* were more susceptible to the same parasite strain (Walshe *et al.* 2011). If ROS are involved in parasite susceptibility, then changes in H_2O_2 levels should reflect this susceptibility of the fly to trypanosomes. Since successful trypanosome establishment and maturation occurs in different tsetse tissues, H_2O_2 levels were measured in midguts and salivary glands of both sexes of tsetse flies. Differences in H_2O_2 levels were found to be not significant between midguts and salivary glands of both sexes, suggesting that higher susceptibility of male tsetse is not due to ROS. H_2O_2 measurements between midguts and salivary glands of males were not statistically different (p-value = 0.617). Larger standard error was observed in salivary glands than midguts, possibly due to the small sample size for H_2O_2 measurements of salivary glands.

Since some salivary glands might emit low ROS levels and some high ROS levels, a larger sample size should balance out the H_2O_2 measurement and lead to smaller error bars. As female flies emerge earlier than males, this could mean that females are exposed to a longer period of starvation compared to males. However, the lasting effects of this starvation period (from emergence to being fed) were not apparent since H_2O_2 measurements in both males and females are comparable in both midguts and salivary glands. It would be interesting to assay trypanosome-infected male and female tsetse to see if differences in H_2O_2 levels between the sexes would become more apparent.

Prior immune-stimulus of tsetse flies leads to lower susceptibility of tsetse to midgut infections with *T. b. brucei* (Hao *et al.,* 2001). Previous work using *Drosophila* and *An. gambiae* have shown that ROS play an important role in host defence against bacterial challenges (Lemaitre and Hoffmann 2007; Molina-Cruz *et al.* 2008). In *Drosophila,* natural infections with bacteria led to a rapid induction of ROS synthesis in the gut (Lemaitre and Hoffmann 2007). Reduction of Duox, a gene involved in the generation of ROS, led to increased susceptibility of *Drosophila* to oral infection of *Erwinia carotovora,* a Gram-negative bacteria (Lemaitre and Hoffmann 2007). Similarly, susceptibility to bacterial infection in *An. gambiae* has been directly correlated with systemic levels of H_2O_2, where female mosquitoes that survive a bacterial infection and are deemed resistant had high H_2O_2 levels, while susceptible female *An. gambiae* had low H_2O_2 levels (Kumar *et al.* 2003). Therefore, to determine whether bacterial infections similarly induced ROS in tsetse, flies were fed live and dead *E. coli* and LPS-free PBS to see if feeding live *E. coli* caused the highest ROS levels. Unexpectedly, ROS levels between the three treatment groups did not differ significantly, suggesting that ROS are not induced upon Gram-negative bacterial challenge in tsetse. Hao *et al.* (2003) hinted at the possibility that intracellular redox levels might act as an effective messenger in the activation of other immune gene products involved in refractoriness since H_2O_2 has been shown to be an effective messenger in activating transcription factor NF-κB (Schmidt *et al.* 1996). The same bacterial-challenge using a Gram-positive bacteria instead (dead *S. aureus*) was repeated. The results indicate that ROS levels do not increase upon this Gram-positive bacterial stimulation. ROS are not induced when tsetse flies are challenged with either Gram-positive or Gram-negative bacteria, suggesting that tsetse flies respond to bacterial challenges using other defence mechanisms such as induction of antimicrobial peptides (Hao *et al.* 2001). A possible explanation for the differences seen between mosquitoes and tsetse flies lies in the fact that *Drosophila* feed on food sources which can be rich in microbes requiring a robust

midgut defence. In contrast tsetse flies are obligate haematophages and thus virtually always feed on a sterile food source. Consequently they may have a reduced requirement for an energetically expensive midgut immune response. Another possible explanation lies in the fact that an elevated immune response to ingested microbes may compromise the symbionts that supply essential nutrients to the fly (Eissa *et al.* 1996; Aksoy 2000; Pais *et al.* 2008). Without the symbionts, the fecundity and longevity of tsetse are severely reduced (Eissa *et al.* 1996; Dale and Welburn 2001; Hao *et al.* 2003).

Since *Wigglesworthia* and *Sodalis* are Gram-negative bacteria and closely related to *E. coli,* we wanted to examine whether symbionts independently generate ROS. However, since we are only able to culture *Sodalis in vitro,* we examined whether *Sodalis* generates ROS compared to its media control. *Sodalis* is believed to increase midgut trypanosome infection in the initial bloodmeal in tsetse (Maudlin and Ellis 1985; Maudlin *et al.* 1990; Dale *et al.* 2001), therefore if ROS are correlated with high susceptibility to trypanosome infections then we would expect low ROS levels. Although the *in vitro* experiment suggests that *Sodalis* does not generate ROS, we are aware of the narrow discriminating power of the *in vitro* experiment compared to *in vivo* work.

As trypanosomes undergo development in the tsetse fly, they are covered by different surface molecules. The transformation of *T. brucei* BSFs into PCFs in the tsetse midgut involves a change in the parasite surface coat, from variant surface glycoproteins (VSG) to procyclins (Roditi *et al.* 2008). Procyclins are major glycosylphosphotidyl inositol (GPI)-anchored proteins that have either C-terminal glu-pro (EP) or gly-pro-glu-glu-thr (GPEET) repeats (Pays and Nolan 1998). The N-terminal domains of procyclins are susceptible to proteolysis, however the amino acid repeats at the C-terminus are not (Acosta-Serrano *et al.* 2001). This suggests possible functions of procyclins in protecting parasites in the fly or their involvement in ligand-signalling of trypanosomes in tsetse. TsetseEP protein, named for the extensive C-terminal region of glutamic acid-proline repeats, shows notable sequence identity to the repeat region of the EP form of procyclin surface coat of *T. b. brucei* (Haines *et al.* 2010). TsetseEP protein is expressed in the midgut and has also been weakly detected in salivary glands (Haines *et al.* 2010). The functional role of tsetseEP protein has been linked to the immune response system since tsetseEP protein is strongly up regulated in tsetse midgut upon injection of live *E. coli* (Haines *et al.* 2005). It has also been linked to protecting the tsetse midgut from trypanosome infections in both *G. m. morsitans* and *G. palpalis palpalis* (Haines *et al.* 2005; Haines *et al.* 2010). *In vivo* reduction

of tsetseEP protein by RNA interference (RNAi) leads to an increase in midgut parasite infection in older flies, thus increasing fly susceptibility (Haines *et al.* 2010). Since the mechanism by which tsetseEP protein influences parasite survival in the midgut is unknown, perhaps ROS levels are influenced by the concentration of tsetseEP protein in the midgut. This could provide an alternate explanation for fly susceptibility other than a direct impact of tsetseEP protein on the parasites. However, there was no difference in H_2O_2 between EP knockdown and control flies, suggesting that tsetseEP does not influence levels of H_2O_2. The tsetseEP knockdown phenotype of increased parasite susceptibility is likely not associated with ROS levels.

Previous reports indicate that puparia of *G. m. morsitans* exposed to a period of elevated temperature produce flies that are more susceptible to parasites (Burtt 1946). ROS levels in midguts of flies kept at temperatures of 26°C (warm) and 30°C (hot) until emergence were compared. Results show no differences in ROS levels between flies that have emerged from puparia exposed to warm or hot temperatures. Therefore, the increased susceptibility of flies emerging from heat shocked puparia is not due to ROS but instead due to other factors. Levels of H_2O_2 in midguts of emerging flies from both groups were almost three times higher than average H_2O_2 levels in flies previously screened (also reared at 26°C). However, one explanation for this discrepancy could be that the flies used were less than six hours old as the puparia were closely monitored to assay flies soon after emergence. Therefore the majority of flies used for this experiment were less than six hours old while baseline H_2O_2 levels were obtained from a mixture of flies up to 24 hours old.

During the course of this investigation, baseline midgut H_2O_2 concentrations in tsetse changed from experiments carried out in April (1.8 mM per mg/ml) and September 2011 (1.29 mM per mg/ml). This may, in part, be caused by fluctuations in humidity in the tsetse colony. Humidity decreased to approximately 52% in September compared to approximately 70% in April. Tsetse flies kept in low humidity usually deplete their water reserves, and therefore metabolize fats in order to maintain water content (Bursell 1956; Popham and Vickers 1979). The stress caused by depletion of water reserves might in turn influence ROS levels. Decrease in H_2O_2 baselines could also be linked to discrepancies between Amplex Red kits, possibly affecting baseline H_2O_2 measurements. Differences may also be attributed to the length of time the samples were left on ice.

The lack of significant differences between experimental and control groups in the experiments could be attributed to the fast rate of dissociation of H_2O_2, particularly in blood where H_2O_2 is only stable for less than one minute (Bocci *et al.* 2005). If the catalase present in tsetse is active then in order to prevent the dissociation of H_2O_2 into oxygen and water by catalase, a catalase inhibitor such as 3-amino-1,2,4-triazole should have been used together with PBS while dissecting tissues in order to delay the dissociation of H_2O_2 and obtain a more accurate reading. The use of this inhibitor has been used in several studies measuring H_2O_2 in mosquito midgut (Pan *et al.* 2011) and hemolymph (Kumar *et al.* 2003; Kumar *et al.* 2010).

ROS have been implicated to play a role against both *Plasmodium* and bacterial infections in *Anopheles* mosquitoes. Strains of *An. gambiae* mosquitoes with higher systemic ROS levels have a better survival rate when exposed to bacteria, while reduction of ROS by supplementing antioxidants in the diet decreased survival, thus suggesting that ROS are an effective immune response against bacteria (Molina-Cruz *et al.* 2008). Molina-Cruz *et al.* (2008) also showed that oxidative stress after a bloodmeal is exacerbated by *Plasmodium* infection. The role of ROS in tsetse refractoriness to trypanosome establishment has been demonstrated indirectly by dietary supplementation of antioxidants (glutathione, cysteine, *N*-acetyl-cysteine, ascorbic acid and uric acid) in a bloodmeal (Macleod *et al.* 2007b). Addition of antioxidants led to a substantial increase in trypanosome infection rates in the midgut of tsetse flies. However, parasite maturation rates (midgut PCFs to mammalian infective metacyclic forms) are more complex: either the midgut's oxidative state plays no part in maturation or a specific oxidant is needed to trigger migration to salivary glands (Macleod *et al.* 2007a). The authors concluded that antioxidants help detoxify free radicals in the midgut, hence promoting trypanosome survival (Macleod *et al.* 2007b). This is in agreement with our only result suggesting a role for ROS in trypanosome infections which is the observation of a significant (p-value = 0.029) increase of 44% in H_2O_2 levels in flies infected with live trypanosomes *(T. b. brucei)* compared to uninfected flies. Previous work has shown that H_2O_2 levels in the tsetse proventriculus increased significantly in the presence of BSFs parasites, while self-cured flies (initially infected but have since cleared the parasites) had lower H_2O_2 levels (Hao *et al.* 2003). Generally, for the first three days following the infected bloodmeal, trypanosomes undergo unrestricted growth in tsetse midgut; however after this time, an attrition phase leads to the complete elimination of infection occuring in a proportion of flies (Gibson and Bailey 2003). Since a significant increase in H_2O_2 levels in flies three days post-infection was observed, perhaps the increase

in ROS is part of the attrition phase that occurs. Hao *et al.* (2003) have suggested that ROS might be involved in clearing trypanosomes early in the infection process either directly or indirectly through the activation of other immune gene products involved in refractoriness. Therefore, we cannot state that H_2O_2 are involved in trypanosome-killing, but rather, ROS are up regulated when flies are exposed to trypanosomes in the bloodmeal.

In this chapter, an indirect approach was used to investigate whether a positive correlation exists between midgut H_2O_2 levels and resistance to trypanosomes. Flies were exposed to various physiological conditions known to impact fly susceptibility and consequently measured H_2O_2 levels in relevant tissues. Indirect evidence suggests that susceptibility of tsetse under diverse physiological conditions is not due to ROS. H_2O_2 levels are significantly higher in flies starved for a week than age-matched fed flies, which is not consistent with a role for ROS in inhibiting trypanosome establishment. In addition, H_2O_2 levels are not influenced by fly sex, flies fed different fractions of a bloodmeal, flies that are exposed to either Gram-positive or Gram-negative bacteria, flies with transcript knockdown of tsetseEP protein and puparial conditions. Moreover, *in vitro* cultured *Sodalis* does not induce ROS levels. Finally, this data shows that H_2O_2 levels increase when flies are exposed to live trypanosomes. The evidence gathered suggest that elevation of H_2O_2 levels by the physiological events investigated does not explain why those events result in changes in susceptibility of the tsetse fly to trypanosomes compared to standard laboratory conditions. However, these results should be carefully interpreted as the lack of a catalase inhibitor during dissection might account for the lack of differences in H_2O_2 levels observed and might reflect an inaccurate measurement of H_2O_2.

The finding that ROS is elevated by trypanosome infections remains an intriguing finding but further experiments will be needed to investigate any link between that elevation and the fate of the trypanosome in the fly midgut. In consequence of the above we decided to investigate the roles of genes involved in ROS generation (Dual oxidase – Duox) and ROS removal (Oxidation resistance 1 – OXR1) in relation to trypanosome susceptibility and the results of this are presented in Chapter 4.

CHAPTER 4

Knockdown of genes involved in homeostasis of reactive oxygen species (ROS)

4.1 Introduction

In eukaryotes, the innate immune system plays an important role in host defence against various microorganisms (Medzhitov and Janeway 1997). An important feature of this defensive response is the production of microbicidal reactive oxygen species (ROS) (Rosen *et al.* 2002). ROS are highly reactive oxygen-derived molecules including free radicals and peroxides. ROS include free radicals such as superoxide anion ($O2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). In *Drosophila,* natural gut infection has been linked to rapid bursts of ROS synthesized by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme, dual oxidase (Duox) (Ha *et al.* 2005a; Ha *et al.* 2005b). In addition to the NADPH domain, Duox proteins have an N-terminal extracellular peroxidase domain that produces ROS in a harmonized manner (Meitzler and Ortiz de Montellano 2009; Welchman *et al.* 2009). Duox swiftly synthesizes ROS in the gut of *Drosophila* following oral ingestion of bacteria (Ha *et al.* 2005a). In *Drosophila,* repression of Duox via gene knockout leads to persistence and proliferation of ingested bacteria in the intestinal tract (Ha *et al.* 2005a).

ROS are generated rapidly as part of an oxidative burst and can then fulfil a range of functions including playing a role in immune response to pathogens (bacteria and parasites) observed in various different insect species (Ha *et al.* 2005b; Leto and Geiszt 2006; Molina-Cruz *et al.* 2008). Since ROS are made up of highly reactive molecules, ROS can react and damage biomolecules including proteins, DNA and lipids, leading to oxidative stress, cell function loss and programmed cell death (Nordberg and Arner 2001). The oxidative burst produced by the rapid generation of ROS may result in self-harm if surplus ROS are not efficiently removed. This redox system of ROS generation and removal is critical in *Drosophila,* because flies which lack the ability to remove ROS (via catalase knockout) experience increased mortality rates (Ha *et al.* 2005b). Therefore, to counteract the toxic effects of ROS, antioxidant defence enzymes are in place to keep ROS levels at an acceptable level (Ha *et al.* 2009a).

The antioxidant defence represents enzymes that detoxify ROS, these include glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD) and thioredoxin peroxidase. Superoxide dismutase reduces $O_2^{-\bullet}$ into H_2O_2, a less reactive species (DeJong *et al.* 2006). Catalase, a tetrameric enzyme in turn transforms H_2O_2 into water and molecular oxygen (DeJong *et al.* 2006). In *Drosophila,* after ROS generation by Duox (induced by microbes), ROS are eliminated by immune-regulated catalase (IRC), a secretory antioxidant enzyme

with catalase activity which removes any remaining luminal ROS which might harm gut epithelia, hence protecting the host from excessive oxidative stress (Ha *et al.* 2005b). High mortality rates have been observed in IRC knocked down flies following natural infection because these flies are unable to remove ROS due to high levels of H_2O_2 (Ha *et al.* 2005b). In *Anopheles gambiae,* the detoxification of ROS has been shown to be an important physiological difference between refractory and susceptible strains of mosquitoes to *Plasmodium* infections (Kumar *et al.* 2003). Refractory strains of *An. gambiae* have increased steady-state ROS levels, which lead to parasite killing in the midgut (Kumar *et al.* 2003). The authors also suggested that catalase is most likely a contender in enhancing *Plasmodium* killing by encapsulation (Kumar *et al.* 2003). Recently, oxidation resistance 1 (OXR1), an ancient gene present in all eukaryotes, was shown to regulate basal levels of the two enzymes that detoxify H_2O_2, catalase and glutathione peroxidase (Elliott and Volkert 2004; Durand *et al.* 2007; Jaramillo-Gutierrez *et al.* 2010). OXR1 was found to protect mosquitoes from oxidative stress and an induction in transcript levels of OXR1 was observed in response to systemic injection of H_2O_2 (Jaramillo-Gutierrez *et al.* 2010). Therefore, the fine homeostatic redox balance is an important event during host-parasite and host-microbe interaction. This is particularly important in tsetse flies as tsetse are obligate blood feeders that ingest enormous bloodmeals averaging 1.7 times the unfed body weight (Munks *et al.* 2005).

One of the constituents of blood include haemoglobin, and the latter consists of four protein subunits, each containing haem with iron being the main molecule (Munks *et al.* 2005). Free haem released during blood digestion generates ROS due to the interaction between haem's iron and oxygen molecule creating the free radical $O_2^{-\bullet}$, which can then be reduced to H_2O_2 and oxygen (Munks *et al.* 2005). However, an even more reactive free radical, the hydroxyl radical ($OH^{\bullet}$) can be produced by the Fenton reaction (Munks *et al.* 2005; Graca-Souza *et al.* 2006). The hydroxyl radical can then react with other molecules and eventually produce highly reactive alkoxyl and peroxyl radicals (Graca-Souza *et al.* 2006). Following a bloodmeal by *An. gambiae* mosquitoes, Duox and a peroxidase form a dityrosine barrier (covalent bonds between tyrosine residues) which decreases midgut permeability to immune elicitors thereby preventing immune activation and creating a suitable environment for bacterial growth and *Plasmodium* development (Kumar *et al.* 2010). However, if this barrier was disrupted, efficient pathogen-specific immune molecules would be elicited, resulting in chronic immune activation and ROS generation

(Kumar *et al.* 2010). Therefore, again the fine balance between ROS generation and ROS removal is essential.

In this chapter, knockdown of Duox, the enzyme that generates H_2O_2, was attempted to subsequently determine the effect on susceptibility of the tsetse fly to trypanosomes. Based on previous work conducted in mosquitoes, the hypothesis that knockdown of Duox results in increased parasite prevalence since reducing Duox should result in decreased H_2O_2 levels was tested. Attempts were also made to clone and knockdown a detoxifying enzyme, immune-regulated catalase (IRC). In addition attempts were made to clone and knockdown another detoxifying enzyme, oxidation resistance 1 (OXR1) and subsequently observe trypanosome susceptibility in flies. The hypothesis in this latter case was that knocking down OXR1 will reduce parasite prevalence since knockdown of OXR1 results in increased H_2O_2 levels. RNA interference (RNAi) involving sequence-specific gene knockdown via injection of double-stranded RNA was used to knockdown these genes.

4.2 Materials and methods

4.2.1 Fly maintenance

Flies were maintained as outlined in section 2.2.1.

4.2.2 Trypanosome stocks

Trypanosoma brucei brucei strain TSW196 bloodstream form (BSF) parasites (Paindavoine *et al.* 1986) were used in trypanosome infection experiments as outlined in section 2.2.2.

4.2.3 RNA extraction from collected tissue

Tsetse flies were dissected for various tissues including midgut, salivary gland, fat body, reproductive tissue (testes), flight muscle, carcass (i.e. fly minus specific organ) and whole fly. Tissues were dissected in PBS, snap frozen in liquid nitrogen and kept at -80°C prior to RNA extraction as outlined in section 2.2.5.

4.2.4 RT-PCR analysis and assessment of transcript abundance

Multiple sets of primers were designed for OXR1 RT-PCR reactions. *G. m. morsitans* GAPDH (Accession number DQ016434) was used as a loading control. Table 4.1 shows the primer sequences of Duox, IRC, OXR1 and housekeeping gene, GAPDH.

The RT-PCR cycling conditions were: 45°C for 45 minutes, 95°C for 2 min, 30 cycles of 95°C for 45 seconds, 55°C for 1 min (59°C for Duox; 59°C for OXR1 with 40 cycles), 68°C for 1 min and a final extension of 68°C for 5 min. RT-PCR products were analyzed by gel electrophoresis using 1.5% agarose gel. Band intensities were measured using Gene Tools software on a Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

Table 4.1. Primer sequences of Duox, IRC, OXR1 and housekeeping gene, GAPDH.

Primers	Primer sequences
Duox Forward	5'- GCCGCTGCTCTCGGCATGAA - 3'
Duox Reverse	5'- GCCCCTTTGCAGTCCAAGCCA - 3'
IRC Forward	5'-ATTGAAATCAACAAATTCTCTA-3'
IRC Reverse	5'-TATTTTCATAAGTGGCATCCAT-3'
OXR1-A Forward	5'- GCTCGAGCAGAAGGCTATTCCTGGT - 3'
OXR1-A Reverse	5' - GGCCGTTTCCGGCACCAACTA - 3'
OXR1-B Forward	5'- GAAATTTTAACCGAAGAGCATCGCGA -3'
OXR1-B Reverse	5'- TGTGGCGCCAAAGGCTCATTG - 3'
OXR1-C Forward	5'- GCATCGCGAAAAACTCTGCAGTCA - 3'
OXR1-C Reverse	5'- GCACGATTGCGAGCGACCCT - 3'
GAPDH Forward	5'-CTCAGCTTCTGTGCGTTG - 3'
GAPDH Reverse	5'-AGAGTGCCACCTACGATG - 3'

4.2.5 Template gene sequences

Blast sequence similarity searches were performed using EST data available from the GeneDB database (http://www.genedb.org). The Duox clone (GMre-16d11.q1k) was selected from the *G. m. morsitans* reproductive EST library, IRC clone (cn787) selected from the fat body library and OXR1 clone (Gmm-2826) selected was from the midgut EST library.

An RT-PCR was first performed on tissues to check for gene expression. Following this, thorax was chosen as the tissue used to clone Duox, midgut chosen as the tissue for IRC and OXR1 cloning. The RT-PCR reaction consisted of 35 cycles for Duox and 30 cycles for IRC and OXR1. This included a reaction of 3 µl template for Duox or 1 µl of midgut template for IRC and OXR1, 12.5 µl Access Mastermix, 1 µl Primer 1(forward), 1 µl Primer 2 (reverse), 0.4 µl AMV (RT) and nuclease free water to amount to a 25 µl reaction. A PCR reaction was then performed on the RT-PCR products using primers shown in Table 4.2. The PCR reaction included 1 µl template, 2.5 µl buffer, 2.5 µl dNTPs, 2.5 µl $MgCl_2$, 1 µl primer 1 (forward), 1 µl primer 2 (reverse), 0.1 µl Taq DNA polymerase and 14.4 µl nuclease free water. PCR cycling conditions included: 94°C for 5 minutes, 30 cycles of 94°C for 1 min, 55°C (IRC) for 1 min (59°C for OXR1; 57°C for Duox), 72°C for 1 min and a final extension of 72°C for 1 min. Both RT-PCR and PCR products were then run on a 1.5% agarose gel.

4.2.6 A-tailing procedure for PCR fragments

The PCR product was subjected to poly-A tailing using Taq polymerase (New England BioLabs) to selectively add adenosine (A) at 3'-ends of the amplified DNA to facilitate the cloning of the PCR product into the vector. The reaction consisted of 6.4 µl of the PCR, 1 µl Taq DNA Polymerase 10X Reaction Buffer, 0.6 µl $MgCl_2$, 1 µl dNTP and 1 µl Taq DNA Polymerase. The reaction mix was to incubate at 70°C for 30 minutes.

4.2.7 Ligation using pGEM-T Easy Vector

The Promega pGEM-T Easy PCR holding vector was used according to manufacturer's instructions. Briefly, 5 µl of 2X Rapid Ligation Buffer was added to 1 µl pGEM-T Easy Vector (50ng/µl), 3 µl of the PCR product, and 1 µl of the T4 DNA Ligase (3 Weiss units/µl) to make a 10 µl standard reaction. The reactions were mixed by pipetting and incubated at 16° for 3 hours.

Table 4.2. Primer pairs, sequences and sizes used for Duox, IRC and OXR1 cloning.

Primer Pairs	Primer Sequences	Size (bp)
Duox-Forward	5' - AAACTCGCGAAAGAAGGCAAAAA - 3'	807
Duox-Reverse	5' -TATGGCGCAAATCTGTATGTTCTG - 3'	
IRC-Forward1	5'-GCGGATGAAGAGCAAAAG-3'	1,232
IRC-Reverse1	5'-CTTGATGTTGACGATCGCAAC-3'	
IRC-Forward2	5'-AGTGGCGAGAAAAATGTTACCC-3'	423
IRC-Reverse2	5'-AGATTCCGATATTCCACCGACTA-3'	
IRC-Forward3	5'-GCGGATGAAGAGCAAAAG-3'	954
IRC-Reverse3	5'-AGATTCCGATATTCCACCGACTA-3'	
IRC-Forward4	5'-AGTGGCGAGAAAAATGTTACCC-3'	701
IRC-Reverse4	5'-CTTGATGTTGACGATCGCAAC-3'	
IRC-Forward5	5'-ATTGAAATCAACAAATTCTCTA-3'	662
IRC-Reverse5	5'-TATTTTCATAAGTGGCATCCAT-3'	
IRC-Forward6	5'-CCAAGCCACGCCCACTCT-3'	775
IRC-Reverse6	5'-ATCCCTCATGAAATTTGCTAACA-3'	
OXR1-Forward	5'- GAAATTTTAACCGAAGAGCATCGCGA -3'	442
OXR1-Reverse	5'- TGTGGCGCCAAAGGCTCATTG - 3'	

4.2.8 Transformations using pGEM-T Easy Vector Ligation Reactions

Ligation reaction mix (2 µl) was added to a pre-chilled 1.5 ml Eppendorf tube followed by the addition of *E. coli* XL1 competent cells (100 µl). Tubes were mixed by gently flicking, and placed on ice for 20 minutes. The cells were then heat-shocked in a water bath at 42°C for 1 minute then placed on ice for 2 minutes. LB broth (250 µl) was added to tubes and left to incubate on a shaker (Grant-Bio SC-20) at 1,150 g for 1 hour at 37°C. After incubation, 100 µl and 150 µl of each transformation culture was plated onto LB Ampicillin (50 µg/ml) with IPTG (30 µl)/X-Gal (20 µl). Plates were left to incubate for 24 hours at 37°C.

4.2.9 Restriction enzyme digestion

A PCR was performed from clones by using single colonies picked from the plates, incubated overnight with 5 ml LB and 10 µl ampicillin. After overnight incubation a 500 µl mixture of each colony and 250 µl LB glycerol was added and mixed by quick vortexing. Tubes were then stored in -80°C. The rest was spun down into a pellet and a plasmid miniprep was done. Plasmid containing the insert was extracted using the Miniprep Spin Kit (Qiagen, California, USA) as outlined in section 2.2.8. Restriction enzyme Eco RI from Promega (Madison, WI, USA) was used for the enzyme digestion as outlined in section 2.2.8. Plasmid DNA was sent for sequencing (DNA Sequencing Core, Molecular Biology Unit, Cardiff University) and resulting sequences were aligned to sequences from GeneDB. After multiple attempts, we were unable to clone IRC, therefore no further work was carried out on IRC. OXR1 was chosen instead, and was therefore cloned.

4.2.10 Synthesis of dsRNA

Double stranded RNA was synthesized as outlined in section 2.2.9. Plasmids of Duox and OXR1 were used as templates for dsRNA synthesis. Primer sequences are listed, with T7 promoter sites underlined in Table 4.3.

Using gene sequences available at the time of the experiment, knockdown constructs were made for both Duox and OXR1 and are shown in Figure 4.1 Panels A and B respectively. PCR cycling conditions included: 94°C for 5 minutes, 30 cycles of 94°C for 1 min, 55°C for 1 min (eGFP and OXR1; 57°C for Duox), 72°C for 1 min and a final extension of 72°C for 1 min. dsRNA was purified using MEGAclear™ columns (Ambion) and eluted in nuclease free water. dsRNA concentrations were measured using a Nanodrop ND-1000 (Wilmington, DE) spectrophotometer. Eluates were concentrated in a Christ (Osterode, Germany) 2-18 rotational vacuum concentrator to obtain dsRNA concentrations of 3 µg/µl.

4.2.11 dsRNA injection

The RNAi technique was optimized by first injecting flies 24 hours after a bloodmeal. A titration experiment was performed with varying concentrations of dsDuox (1.5 µg, 3 µg, 4.5 µg, 6 µg) in order to check for gene knockdown and mortality rates (1 hr, 24 hrs, 48 hrs, 72 hrs and 96 hrs post-injection). A timecourse experiment on tsetse midguts was carried out after optimal concentration of dsRNA was chosen. Transcript expression was measured in midguts of flies at days 1, 3, 5 and 11 post-dsDuox (6 µg) injection. Similarly, transcript expression was measured in midguts of flies at days 1, 3, 7 and 10 post-dsOXR1 (6 µg) injection and at day 7 post-dsOXR1 (9 µg) injection. To check for knockdown, tissues were dissected, RNA extracted & RT-PCR conducted. Products were then run on a 1.5% agarose gel. Band intensities were measured using Gene Tools software on a Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

Table 4.3. Primer sequences for dsRNA synthesis of Duox, OXR1 and eGFP control with T7 promoter sites underlined.

Primers	Primer Sequences
T7Duox-F	5'- TAATACGACTCACTATAGGGTTGCGAGTGCCCAATGACCACG - 3'
T7-Duox-R	5'- TAATACGACTCACTATAGGGTGGGGCCTTTCCTGCATTGGCT - 3'
T7-OXR1-F	5'- TAATACGACTCACTATAGGGGCATCGCGAAAAACTCTGCAGTCA - 3'
T7-OXR1-R	5'- TAATACGACTCACTATAGGGGCACGATTGCGAGCGACCCT - 3'
T7-eGFP-F	5'-TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTTC - 3'
T7-eGFP-R	5'- TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGCC - 3'

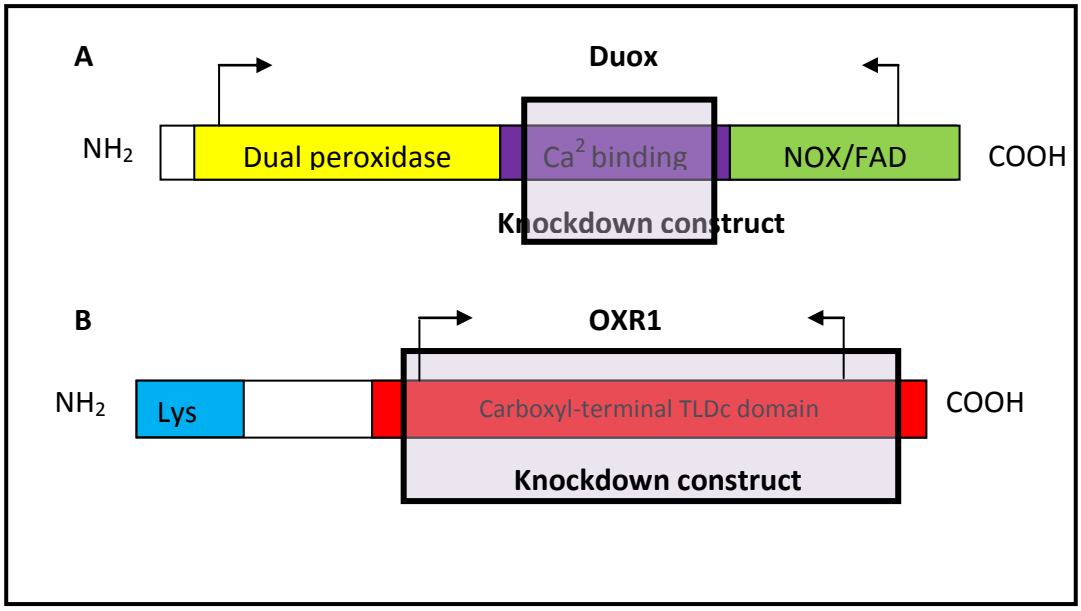


Figure 4.1. Schematic representation of knockdown construct in relation to protein domains of dual oxidase (Duox) and oxidation resistance 1 (OXR1). (A) Protein domains of Duox and knockdown construct encompassing sequence available at the time of experiment. **(B)** Protein domains of OXR1 and knockdown construct. Arrows indicate forward and reverse primers for RT-PCR.

4.2.12 Trypanosome infections

All flies were fed a regular bloodmeal on the day of emergence. Flies were then injected with 2 µl of 3 µg/µl of dsRNA (Duox and eGFP) and a non–injected control group. All flies were then fed a trypanosome infected bloodmeal 24 hours later, and flies that did not feed were removed 24 hours later. Midguts of flies were dissected a week after having fed on the infected bloodmeal. A light microscope (125 X total magnification) was used to score parasite infection. Midguts of flies were dissected 72 hours post-infection, shredded into 100 µl of saline on a glass slide and infection status was scored by searching for motile trypanosomes in 10 random fields using dark field microscopy (125 X magnification). Midguts were collected in liquid nitrogen, and RT-PCRs were performed on tissues to check whether genes were knocked down.

4.2.13 Hydrogen Peroxide (H_2O_2) assay to measure ROS levels

An Amplex Red Hydrogen Peroxide Kit from In Vitrogen (A22188) was used to measure H_2O_2 levels in dissected tsetse midguts as outlined in section 3.2.3.

4.2.14 Quantifying Samples using Bradford Assay

H_2O_2 levels in dissected tsetse midguts were normalized for protein content using the Bradford Assay as outlined in section 3.2.4.

4.2.15 Immunoblotting

Immunoblotting using Hybond^{TM}-P polyvinylidene difluoride (PVDF) transfer membrane (Amersham Biosciences, Amersham, UK) was performed as previously described (Beecroft *et al.* 1993). Two blots were developed with different antibody concentrations. In brief, the first blot was made of 1:3000 dilution of a primary antibody of anti-OXR1 polyclonal rabbit antiserum raised against a region within amino acids 531 and 671 of OXR1 (GeneTex, CA, USA). The secondary antibody was a 1:20,000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG/IgM (ThermoScientific, Rockford, IL). The second blot consisted of 1:1500 dilution of primary antibody of anti-OXR1 polyclonal rabbit antiserum and 1:10,000 dilution of the secondary antibody of horseradish peroxidase conjugated goat anti-rabbit IgG/IgM. The western blot was developed with SuperSignal Dura chemiluminescence substrate (Pierce Chemical Company, Rockford, IL) and Kodak Biomax MR film (Eastman Kodak Company, Rochester, NY) was used to detect chemilunescence. Proteins were stained on PVDF membrane with 0.2% (w/v) nigrosine in

PBS. The exposed film was superimposed onto the stained PVDF membrane to reveal the precise location of the immunoreactive protein bands. A mouse short OXR1 recombinant His-tagged protein encompassing the TLDc domain (the most highly conserved region) was used as a positive control (kind gift from Peter Oliver, University of Oxford). Two different antibody combinations were used for the positive control: (1) 1:5000 dilution of monoclonal anti-polyHistidine primary antibody produced in mouse (Sigma, St. Louis, MO) and a 1:50,000 dilution of the secondary mouse horseradish peroxidase conjugated anti-goat IgG antibody (ThermoScientific, Rockford, IL) or (2) 1:2500 dilution of the mouse monoclonal anti-polyHistidine primary antibody and 1:25,000 dilution of the secondary mouse horseradish peroxidase conjugated anti-goat IgG antibody.

4.2.16 Statistical analysis

Statistical analysis was performed using SPSS16 (SPSS Inc., Chicago, Illinois). Student's t-test was performed to determine if significant differences were present between two groups and ANOVA test was used for multiple comparisons. Differences were deemed significant if a p-value was less than 0.05.

4.3 Results

4.3.1 Localization of Duox and OXR1 transcript expression in tsetse

Transcript expression of Duox and OXR1 was measured by RT-PCR to determine the presence of transcripts in different tissues. Duox and OXR1 transcripts were detected in all tissues investigated (Figure 4.2). Transcript expression of these genes in the midgut is important since this is the site in which trypanosomes become established when first entering the fly.

4.3.2 Response to gene knockdown using RNA interference

RNA interference (RNAi) was used as a tool to transiently reduce Duox mRNA and protein levels in adult male tsetse flies. In order to determine the optimal concentration of dsRNA needed to achieve maximum knockdown with minimal mortality rates, flies were injected with differing concentrations of dsRNA ranging from 1.5 μg to 6 μg (Table 4.4). 6 μg of dsDuox was chosen as the optimal concentration of dsRNA needed to achieve knockdown (approximately 82%) with minimal mortality rates (11%). Another experiment was carried out to determine the length of knockdown. Figure 4.3 Panel A shows that knockdown of Duox transcript occurred slowly, increasing from day 3 (70.5% knockdown), peaking at day 5 (87.6% knockdown) and persisting up to day 11 post-injection (47.4% knockdown). Comparative analysis of normalized densitometry data showed higher transcript knockdown in dsDuox injected than dseGFP injected flies (Insert in Figure 4.3 Panel A). Figure 4.3 Panel B shows the reduction of Duox transcripts in midguts (97.2% knockdown ± 2.8; p=0.018) and reproductive tissues (71.8% knockdown ± 2; p=0.035) 7 days post-injection.

Since 6 μg of dsRNA is the average concentration used to inject tsetse flies in the laboratory for a range of genes, 6 μg of dsOXR1 was chosen to inject flies initially. Midguts were subsequently dissected days 1, 3, 7 and 10 post-injection and semi-quantitative RT-PCR analysis was carried out on the samples (Figure 4.4 Panel A). Injecting 6 μg of dsOXR1 failed to achieve transcript knockdown of OXR1. The concentration of dsRNA was increased to 9 μg but this also failed to acheive knockdown (Figure 4.4 Panel B). The dsRNA concentration could not be increased further due to high mortality rates (Figure 4.4 Panel C). The western blots unfortunately resulted in no signal due to possible weak affinity binding, and were therefore inconclusive.

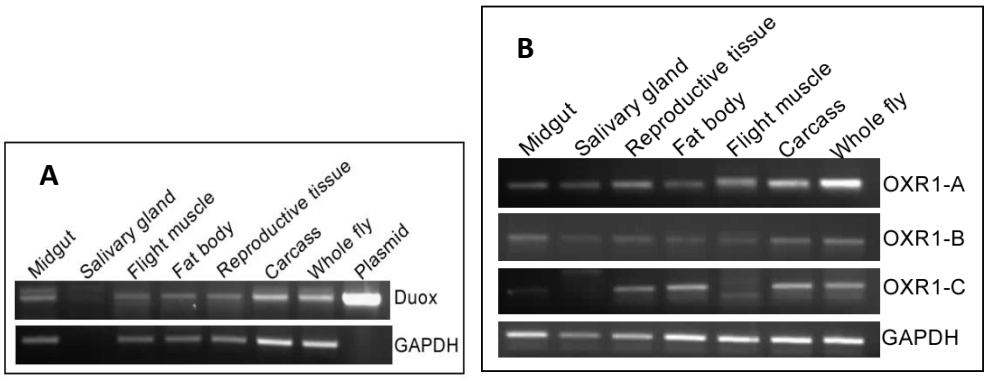


Figure 4.2. RT-PCR showing expression of dual oxidase (Duox) and oxidation resistance 1 (OXR1) and housekeeping gene (GAPDH) in different tsetse tissues. (A) Transcript expression of Duox with a cloned plasmid as a control. **(B)** Transcript expression of OXR1 with 3 OXR1 primer sets: OXR1-A, OXR1-B, OXR1-C.

Table 4.4. Transcript knockdown (highlighted in grey; top half of box) in midgut and reproductive tissue and mortality rates (bottom half of box) following injection with different concentrations of dsDuox.

	1.5 µg	3 µg	4.5 µg	6 µg
Midgut	25% / 10%	52% / 10%	65% / 20%	82% / 11%
Reproductive Tissue	62.5% / 10%	76.4% / 10%	81.15% / 20%	82.5% / 11%

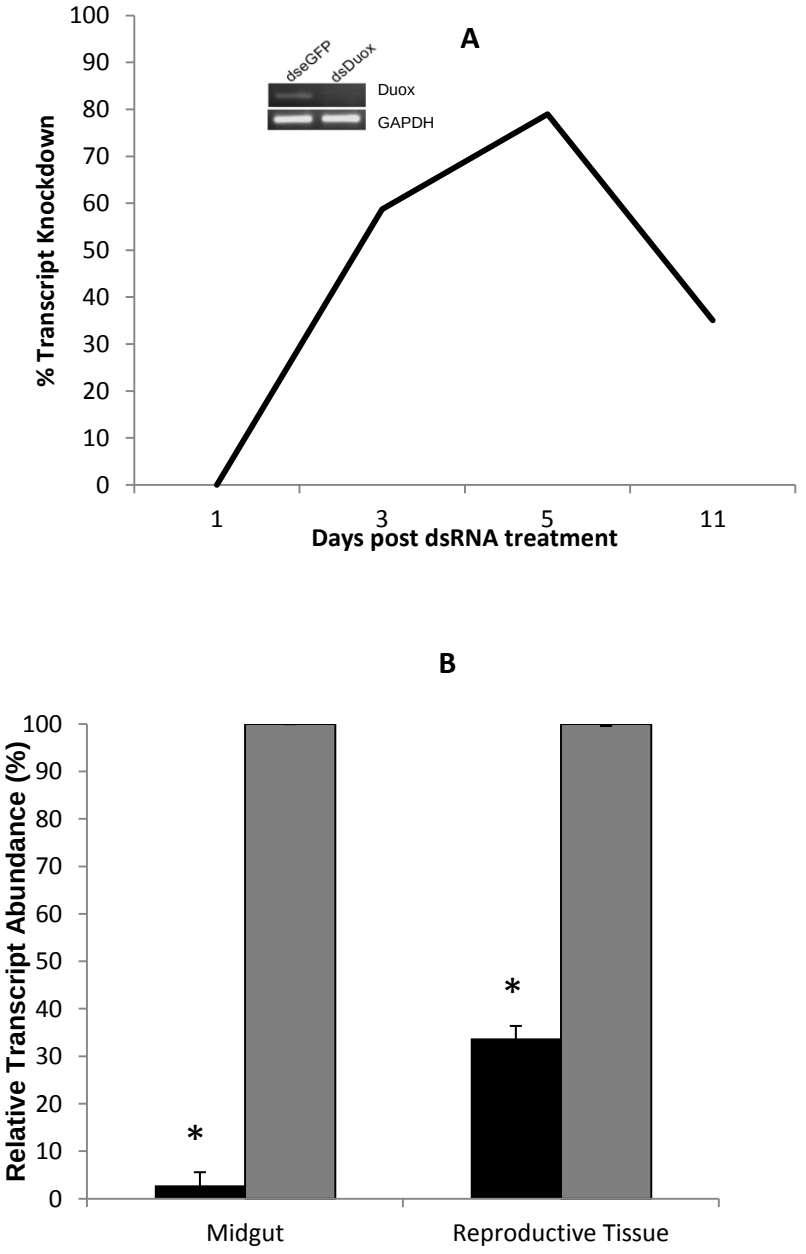
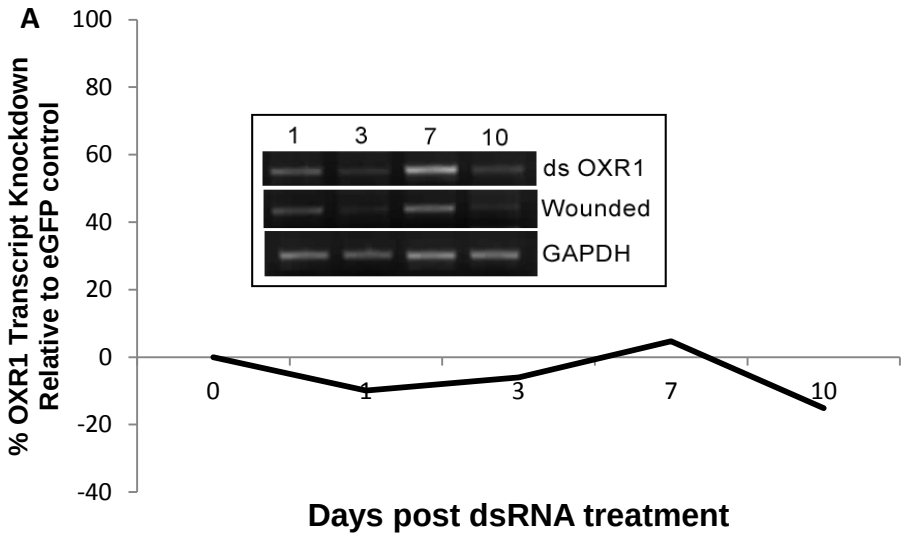


Figure 4.3. Transcript knockdown of Duox following injection with dsDuox. (A) Semi-quantitative RT-PCR analysis of Duox expression following injection of dsDuox (6 µg)(solid black line) or a control dseGFP (dotted black line). Data are presented as the mean knockdown (%) of Duox transcript (y-axis) against the number of days after injection of dsRNA (x-axis). Band intensities were normalized separately for each lane using the corresponding GAPDH loading control band intensities. Insert: Transcript levels in midguts on day 7 post-injection, determined by semi-quantitative RT-PCR. **(B)** Knockdown efficiency on day 7 post-dsDuox in tsetse midguts and reproductive tissues (testes) as determined by densitometry from RT-PCR, normalized against the housekeeping gene, GAPDH; black: dsDuox injected, grey: wounded control; midgut p-value: 0.018; reproductive tissue p-value: 0.035; n=157; mean ± SE for three biological replicates.



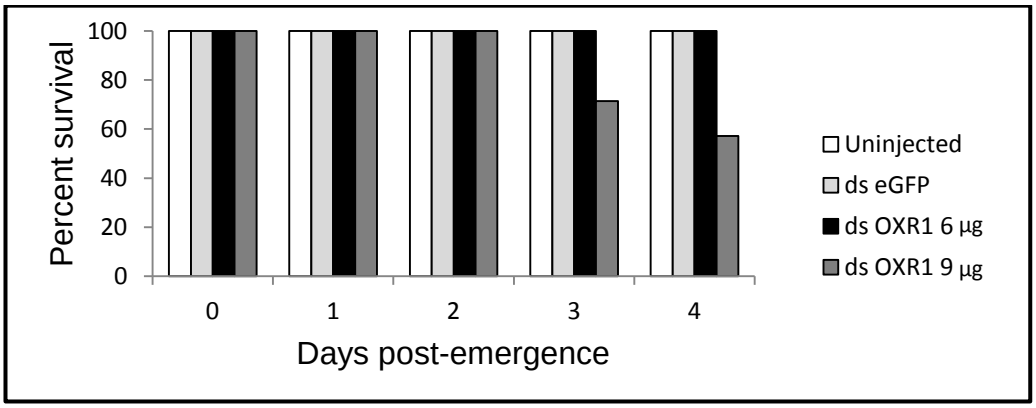


Figure 4.4. Transcript knockdown of OXR1 following injection with 6 µg and 9 µg of dsRNA. (A) Semi-quantitative RT-PCR analysis of OXR1 expression following injection of dsOXR1 (6µg)(solid black line) or a control dseGFP (dotted black line) at days 1, 3, 7 and 10 post-injection. Data are presented as the mean knockdown (%) of OXR1 transcript (y-axis) against the number of days after injection of dsRNA (x-axis). Band intensities were normalized separately for each lane using the corresponding GAPDH loading control band intensities. Insert: Semi-quantitative RT-PCR analysis of OXR1 expression in midguts at days 1, 3, 7 and 10 post-injection of dsOXR1 or wounded flies. **(B)** Semi-quantitative RT-PCR analysis of OXR1 expression 7 days post-injection with 9 µg of dsOXR1 and a wounded control. **(C)** Survival of flies after injection with 9 µg dsOXR1 compared to 6 µg dsOXR1, dseGFP and uninjected group.

4.3.3. Parasite prevalence after dsDuox injection

Since Duox generates H_2O_2, knocking down Duox should result in decreased levels of ROS, giving an opportunity to determine if decreased ROS makes the midgut environment more friendly to parasite proliferation. Flies were thoracically injected with 6 μg of dsDuox after the first bloodmeal and subsequently fed a parasite-infected bloodmeal (*T. b. brucei* TSW196 BSF). A significant increase in midgut infection prevalence was observed in flies injected with dsDuox (38.8% ± 6.6) compared to both the injected control, dseGFP (24.3% ± 3.4; $p<0.05$) and the uninjected control (12.9% ± 0.7; $p<0.001$) (Figure 4.5 Panel A). In all three groups, overall parasitaemia intensity (%) was high, with the majority of flies having more than 100 parasites per field (Figure 4.5 Panel B). Mortality rates were minimal, with no significant differences across the three groups.

The average midgut infection prevalence at the 5th bloodmeal is typically less than 10% (Haines *et al.* 2010). Flies were fed four uninfected bloodmeals, then thoracically injected with dsDuox. At the 5th bloodmeal, flies were fed a trypanosome infected bloodmeal. Midguts were dissected 7 days later (Figure 4.5 Panel C). No differences between dsDuox injected group and control groups were observed. Control groups were consistent with normal rates observed at 5th bloodmeal with less than 10% midgut infection prevalence.

4.3.4 H_2O_2 levels following transcript knockdown of Duox

Since no antibodies were available to check for Duox protein knockdown, H_2O_2 levels in midguts were measured directly following dsRNA injection. Since Duox is known to generate H_2O_2, injecting dsDuox should result in knockdown of Duox protein and lead to decreased H_2O_2 levels but no differences were seen (Figure 4.5 Panel D; dsDuox 1.88 mM ± 0.85 compared to dseGFP 1.77 mM ± 0.19 and uninjected control 1.72 mM ± 0.67). This shows that although injecting 6 μg of dsDuox led to transcript knockdown in the midgut (97.2% ± 2.8), it is possible that the H_2O_2 assay failed to accurately measure H_2O_2 levels due to the short lived nature of H_2O_2. It seems likely that the Duox protein was knocked down since we observed a clear phenotype of increased parasite prevalence. However further work is needed to confirm the role of Duox in parasite infectivity. An alternative explanation may involve other means of H_2O_2 production in the midgut and these compensate for H_2O_2 not being produced by Duox. Consequently the current protocol used for this H_2O_2 assay is inadequate to test for knockdown of enzymatic levels of Duox.

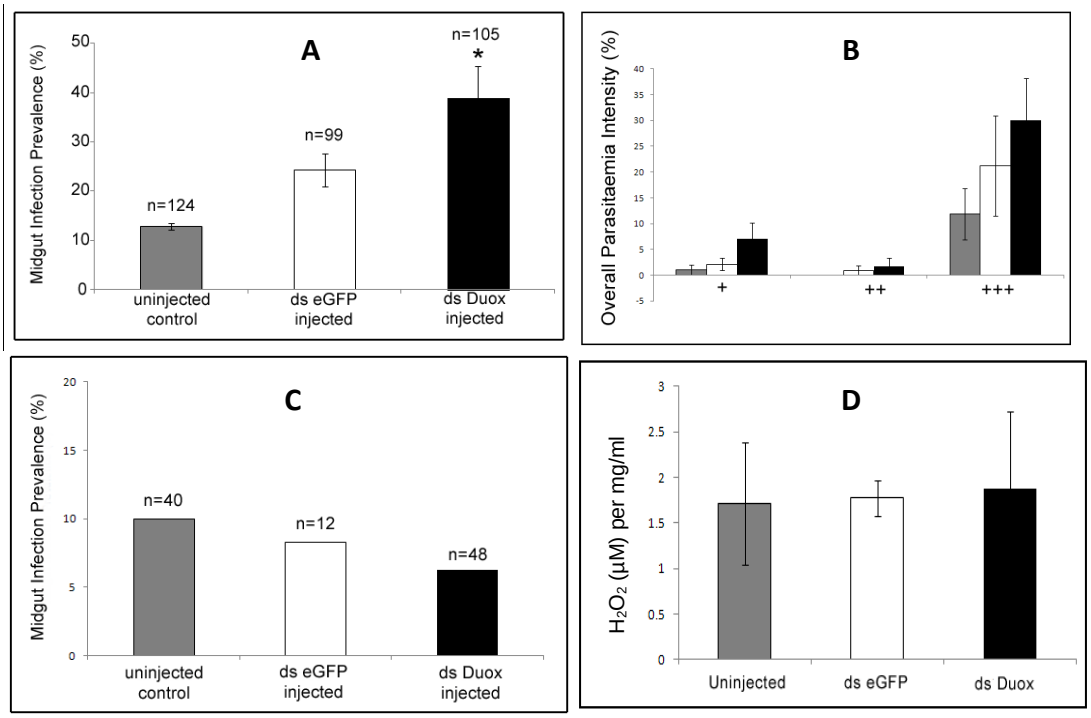


Figure 4.5. Parasite prevalence in the midgut after Duox knockdown and H_2O_2 concentrations (µM) per mg/ml after dsRNA injection. **(A)** Early Duox repression: flies were injected with dsDuox prior to feeding on infected blood with *T. b. brucei*; p-value between the two injected groups<0.05; p-value between uninjected and Duox injected<0.001. n=328; mean ± SE for three biological replicates. **(B)** Overall parasitaemia intensity (%) in flies injected with dsDuox prior to feeding on infected blood with *T. b. brucei*; mean ± S.E. for three biological replicates. Uninjected control (grey), dseGFP injected (white) and dsDuox injected (black), +: <10 parasites/field, ++: 11-99 parasites/field, +++: >100 parasites/field. **(C)** Flies were injected with dsDuox after the 4th regular bloodmeal, then fed on infected blood with *T. b. brucei* at the 5th bloodmeal. This experiment involved one replicate only. **(D)** H_2O_2 concentrations (µM) per mg/ml from midguts of flies 5 days after injection with dsDuox, dseGFP and uninjected control. Mean ± SE for three biological replicates. H_2O_2 levels are represented as concentration (µM) but can be converted by multiplying concentrations by 25 µl (volume of reaction) X 100 (1 µM = 100 pmoles per µl) and dividing by 30 (time of incubation); ie. a factor of 83.33.

4.4 Discussion

Gene knockdown by RNA interference (RNAi) is a valuable tool to study the effects of loss of gene function. Following sequence specific double stranded RNA (dsRNA), mRNA is degraded leading to specific post-transcriptional gene knockdown (Fire *et al.* 1998). The RNAi process includes the introduction of long dsRNA which is subsequently digested by an RNA III-like enzyme (called Dicer in *Drosophila*), into short, interfering RNA (siRNA of approximately 22 bp in length) (Bernstein *et al.* 2001; Siomi 2009). The siRNA then combines with an RNA-induced silencing complex (RISC) which then cleaves mRNA with the homologous sequence to the siRNA (Meister and Tuschl 2004).

In this chapter, attempts were made to knock down genes involved in ROS generation and removal. Dual oxidase (Duox) protein contains an N-terminal extracellular peroxidase domain that can produce ROS (Welchman *et al.* 2009). Two distinct pathways have been suggested to regulate Duox expression in *Drosophila*. First, Duox protein is regulated through a Gαq/phospholipase-Cβ pathway which releases intracellular calcium from the endoplasmic reticulum (Ha *et al.* 2009b). Flies lacking Duox, Gαq or phospholipase-Cβ were killed when exposed to yeast in the diet under normal conditions, therefore this pathway is necessary to control microbes in the diet (Ha *et al.* 2009b). In response to pathogenic microbes, a second p38-MAPK pathway downstream of peptidoglycan receptor PGRP-LC and IMD (not downstream) involves the transcriptional regulation of the Duox gene (Ha *et al.* 2009b). Under normal conditions, phospholipase-Cβ reduces Duox expression, when exposed to pathogenic microbes, it acts upstream of the p38-MAPK pathway to increase Duox expression (Ha *et al.* 2009b). Therefore, this regulation of Duox allows *Drosophila* to efficiently fight off infection while tolerating commensal microbes, therefore achieving a balance with microbes in the gut (Ha *et al.* 2009b).

Since transcript knockdown of Duox in the midgut (and reproductive tissue) of tsetse determined by semi-quantitative RT-PCR was successfully achieved, flies were infected with trypanosomes to determine whether the same phenotype of increased parasites exists in tsetse. We wished to test the hypothesis that lowered H_2O_2 levels would lead to increased midgut trypanosome prevalence. Significant differences were observed in flies injected with dsDuox compared to the control groups. This suggests that the inhibition of Duox in the tsetse midgut promotes trypanosomes establishment. Likewise this suggests that when Duox is present in the midgut, the generation of H_2O_2 promotes the killing of trypanosomes. This is similar to the potential but unproven role of NOS as mentioned in

chapter 2, and it is would be interesting to examine whether reactive oxygen species such as H_2O_2 and reactive nitrogen species such as nitric oxide act synergistically to kill parasites through the production of peroxynitrite.

The significant increase in trypanosome infection in tsetse midgut following knockdown of Duox suggests that reduction of Duox via RNAi should decrease H_2O_2 levels in the midgut, thereby enabling parasites to establish and proliferate. Therefore, to confirm that transcript knockdown of Duox results in decreased levels of H_2O_2, H_2O_2 levels were measured following injection of dsDuox. Surprisingly, no reduction in H_2O_2 levels were observed in flies injected with dsDuox. Since a phenotype of increased parasite prevalence was observed, it seems likely that the Duox protein was knocked down, however H_2O_2 levels remained the same in dsDuox injected flies and control flies.

An explanation may be that other means of H_2O_2 production are present and the systems are therefore redundant. However, the most likely explanation involves the short lived nature of H_2O_2 in blood, which is less than 1 min (Bocci *et al.* 2005). The methodology undertaken for this assay did not consider the fast dissociation of H_2O_2 into oxygen and water by catalase. Hence at the time of the assay, H_2O_2 levels would be basal due to the effects of catalase from the time of dissection to the time of the assay. This is consistent with no differences seen in dsDuox injected flies. Although at the time of the experiment, no catalase gene was found in the GeneDB database which consists of ESTs, the newly released genome indicates the presence of a catalase gene. Hence, in retrospect, tsetse midguts should have been dissected in PBS mixed with a catalase inhibitor such as 3-amino-1,2,4-triazole to delay the dissociation of H_2O_2 by catalase which might be active in tsetse, allowing for a more accurate measurement of H_2O_2, as shown in mosquitoes (Kumar *et al.* 2003; Kumar *et al.* 2010; Pan *et al.* 2011).

Although at the time of the experiment, the only available Duox sequence included the calcium binding domain and thus represents the cloned region, we confirmed that there was no sequence homology to other genes apart from Duox by using the newly available tsetse genome sequence.

Attempts were also made to clone immune-regulated catalase (IRC; cn786) with the hope of subsequently using the clone as a template to synthesize dsRNA for gene knockdown. However, cloning IRC was unsuccessful, possibly due to the short sequence that was available at the time from GeneDB. However, with the recent release of the *Glossina*

genome which was not available at the time of the experiment, no IRC gene has been found in the tsetse genome.

Knockdown of OXR1 was attempted using 6 µg and 9 µg of dsRNA which resulted in high mortality rates due to the high concentration injected into the fly. This was not due to the effect of successful gene knockdown and the resulting phenotype in the fly because semi-quantitative RT-PCR showed that no knockdown was achieved in midguts. Unfortunately, the immunoblot analysis proved to be inconclusive due to no detection of any signal. Therefore the role of OXR1 in relation to parasite susceptibility could not be determined. No attempts were made to inject tsetse with an even higher concentration (>9 µg dsRNA) into the fly since 9 µg of dsRNA resulted in high mortality rates. To overcome high mortality rates, the possibility to administer 9 µg of dsRNA orally with the bloodmeal could be attempted as immunoblot analysis was inconclusive.

Transcript knockdown of Duox did not equate to protein knockdown of Duox as measured by H_2O_2 levels. However, transcript knockdown of Duox resulted in increased infection prevalence in tsetse midguts, suggesting that Duox may play a role against trypanosome infection in tsetse. This result may also suggests a flaw in the H_2O_2 assay technique, and use of a catalase inhibitor might resolve the discrepancy in no differences in H_2O_2 and the resulting phenotype of midgut infection prevalence.

CHAPTER 5

A bioinformatics approach to characterizing antioxidant genes in tsetse

5.1 Introduction

Insects have a competent innate immune system enabling them to distinguish and successfully eliminate pathogens (Hoffmann *et al.* 1999). Tsetse flies, are the cyclical vectors of trypanosomes and are responsible for African trypanosomiasis in humans and animals (Aksoy 2003). The success or failure of infection and maturation of trypanosomes in tsetse are determined by several factors including fly immunity factors (Lehane *et al.* 2003). Although extensive research has been performed on trypanosome biology, lack of genetic information regarding tsetse flies has limited the progress in our understanding of tsetse-trypanosome interactions. Transcriptome sequencing is an appropriate alternative to whole genome sequencing because of its low cost and its ability to offer information about the transcribed portions of genes (Tariq *et al.* 2011). Next-generation Roche 454 sequencing allows faster highly parallel DNA sequencing, and increased sequencing depth of coverage while reducing time, labour and costs (Tariq *et al.* 2011). Differential gene expression analysis of whole transcriptomes with differing phenotypes such as uninfected and self-cured tsetse flies can also be performed using Roche 454 sequencing.

Genome-wide analyses of immune genes and gene families have been conducted in dipterans and show the sharing of many orthologous immune genes with noticeable diversification (Christophides *et al.* 2002). Since different dipteran insects are commonly under different selective pressures from various pathogens in different ecological and physiological settings, it is perhaps not surprising to see the surplus of gene modifications and expansions which have been seen to occur (Christophides *et al.* 2002). The combination of sequencing information and gene knockdown techniques such as RNA interference (RNAi) provides a strong functional analysis platform for insect genes involved in immunity. Sequence analysis of the tsetse fly *Glossina morsitans morsitans* is represented by expressed sequence tags (EST) available on the Sanger *G. m. morsitans* GeneDB database (Lehane *et al.* 2003; Attardo *et al.* 2006; Alves-Silva *et al.* 2010). Although ESTs on numerous tsetse genes are available from tsetse tissue libraries (Lehane *et al.* 2003; Attardo *et al.* 2006), many genes are putative, and not full-length sequences. The lack of full-length sequences might exclude the important domains necessary for a gene's function.

This chapter reports phylogenetic analysis of the tsetse immune genes discussed in the previous chapters, NOS (Chapter 2), Duox and OXR1 (Chapter 4) compared to other invertebrates and vertebrates. A 454 platform was used to compare cDNA sequencing

between uninfected and infected/self-cured tsetse flies. Attempts were made to identify potential genes associated with the trypanosome infection process by comparing expression profiles of the two data sets of flies. Finally, newly available resources were used in an attempt to extend the sequences of NOS, Duox and OXR1 to incorporate necessary domains indispensable for gene functionality.

5.2 Materials and methods

5.2.1 Phylogenetic analysis

Nucleotide sequences for NOS, Duox and OXR1 were chosen from the EST libraries (http://www.genedb.org). NOS was selected from the salivary gland EST library which was assembled from 27,426 EST sequences. Duox was selected from the reproductive tissue EST library assembled from 3,438 EST sequences and OXR1 was selected from the midgut EST library which was assembled from 21,662 EST sequences. The following EST contigs were chosen for the three genes: NOS (GMsg-7530 and ADD18407.1), Duox (GMre-16d11.q1k) and OXR1 (Gmm-2826). The sequences were analyzed for open reading frames (ORFs) (http://www.ncbi.nlm.nih.gov/projects/gorf/) and translated into amino acid sequences. The predicted protein sequences of *G. m. morsitans* of the three genes were aligned using ClustalW to the corresponding genes from multiple species obtained from GenBank NCBI and alignments were manually modified using JALVIEW. Table 5.1 lists accession numbers of NOS, Duox and OXR1 from different species.

The maximum likelihood model with the lowest Bayesian information criterion was chosen since it best describes the substitution pattern. The best selected model for NOS analysis was that of Whelan and Goldman with a gamma distribution with invariant sites (WAG+G+I) (Whelan and Goldman 2001). The best selected model for Duox was the Jones, Taylor and Thornton (JTT) model (Jones *et al.* 1992) with a gamma distribution (JTT+G), and the best model for OXR1 was also the JTT+G model (Whelan and Goldman 2001). Branch support values were analyzed with 1000 bootstrap replicates under neighbour-joining algorithms using MEGA 5 (www.megasoftware.net). Tree was constructed using the program FIGTREE v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 5.1. List of NOS, Duox and OXR1 sequences from different species and their accession numbers.

Gene name	Accession number	Size (amino acids)	Origin
NOS, salivary gland	GMsg-7530	247	*G. m. morsitans*
NOS	ADD18407.1	492	*G. m. morsitans*
NOS	gi|157124093|	1112	*Aedes aegypti*
AGAP008255-PA	gi |31222692|	1112	*Anopheles gambiae str. PEST*
NOS	gi|8473620|	1247	*An. stephensi*
NOS	gi|61175210|	1143	*Apis mellifera*
NOS	gi|170028305|	719	*Culex quinquefasciatus*
GK23979	gi|195433302|	1356	*Drosophila willistoni*
GA19805	gi|125984610|	1348	*D. pseudoobscura*
GH13337	gi|195050934|	1359	*D. grimshawi*
GL26383	gi|195161643|	1121	*D. persimilis*
NOS	gi|6707649|	1244	*D. melanogaster*
Inducible NOS	gi|2935553|	1147	*Homo sapiens*
NOS	gi|3372750|	1206	*Manduca sexta*
NOS , putative	gi|241238513|	1098	*Ixodes scapularis*
NOS salivary gland, putative	gi|242008693|	1104	*Pediculus humanus corporis*
Inducible NOS	gi|1304200|	1147	*Rattus norvegicus*
Dual oxidase putative	GMre-16d11.q1k	432	*G. m. morsitans*
Dual oxidase 1	gi|157116387|	1486	*Ae. aegypti*
AGAP009978-PA	gi|158298988|	1485	*An. gambiae str. PEST*
PREDICTED: similar to CG3131-PA	gi|110755087|	1492	*Ap. mellifera*
Dual oxidase 1	gi|170033274|	1482	*Cx. quinquefasciatus*
Dual oxidase	gi|281364292|	1537	*D. melanogaster*
GM18195	gi|195342093|	946	*D. sechellia*
Dual oxidase 2 precursor	gi|132566532|	1548	*H. sapiens*
Dual oxidase 1	gi|241624918|	1532	*I. scapularis*
Dual oxidase 1 precursor	gi|242018811|	1441	*P. h. corporis*
Dual oxidase 2 precursor	gi|13162322|	1517	*R. norvegicus*
Putative SD08996p	Gmm-2826	297	*G. m. morsitans*
Nucleolar protein c7b	gi|157131340|	1287	*Ae. aegypti*
AGAP001751-PA	gi|158301667|	1129	*An. gambiae str. PEST*
PREDICTED: similar to l(3)82Fd CG32464-PK, isoform K isoform 1	gi|110756598|	1195	*Ap. mellifera*
Nucleolar protein c7b	gi|170056214|	209	*Cx. quinquefasciatus*
Isoform B	gi|21355123|	1270	*D. melanogaster*
Oxidation resistance 1	gi|21618514|	758	*H. sapiens*
Nucleolar protein c7c, putative	gi|241751830|	803	*I. scapularis*
Nucleolar protein c7c, putative	gi|242020183|	913	*P. h. corporis*
Oxidation resistance 1, isoform CRA_a	gi|149066454|	785	*R. norvegicus*

5.2.2 Differential gene expression from tsetse midgut 454 transcriptome data

A tsetse midgut 454 library (cDNA prepared by Stella Lehane using SMARTer PCR cDNA Synthesis kit by Clontech and sequenced and assembled by Alistair Darby using 454 sequencing technology at the Genomics Suite, University of Liverpool) was generously provided to analyze differential expression of genes. The transcriptome data consisted of a combination of naive uninfected flies (fed normal bloodmeal throughout) and flies infected with *T. b. brucei* TSW196 (fed at fourth bloodmeal), and dissected three days later.

The edgeR package (Robinson *et al.* 2010) was used to perform a tagwise dispersion analysis (by Alistair Darby) to reveal 184 differentially expressed genes ($P < 0.05$) between the two midgut libraries (flies fed normal blood and flies fed trypanosome-infected blood). Top-ranking genes (highest fold-change) from the set of 184 were then narrowed down to 70 using arbitrary cut-off values of 1.15 for down regulated genes and 1.2 for up regulated genes (by Alistair Darby). Accession numbers made available were used to retrieve protein sequences of differentially expressed genes from NCBI (http://www.ncbi.nlm.nih.gov/sites/batchentrez). Gene Ontology annotation was performed using BLAST2GO (Conesa *et al.* 2005) by transferring each sequence using BLASTp hits to proteins in NCBI with a significance above $e=1^{-3}$. Various parameters were used by BLAST2GO to return the most likely GO terms. In addition to NOS, Duox and OXR1, other redox genes were searched in the dataset (Table 5.2).

5.2.3 Gene sequence extension using 454 transcriptome data

Antioxidant genes from *D. melanogaster* were selected from FlyBase (http://flybase.org/). Stand alone BLAST searches were performed for these genes against the midgut cDNA data to obtain corresponding contigs. After the contigs were identified, nucleotide sequences of *D. melanogaster* antioxidant genes were aligned to the contigs using ClustalW. Corresponding contigs (nucleotide sequences) were also translated into amino acid sequences by first using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) and subsequently aligned to the protein sequence of the corresponding gene from *D. melanogaster.* Table 5.2 lists the antioxidant genes which were attempted for sequence extension.

Table 5.2. Antioxidant genes, accession numbers of *Drosophila* homologs and the corresponding contigs retrieved via stand alone BLAST searches against the midgut cDNA 454 dataset.

Gene name	Accession number	Homolog from *Drosophila* species	Corresponding contig in midgut cDNA 454 data
Adenylate kinase	gi|24663204|	*D. melanogaster*	3835
Alcohol dehydrogenase	gi|78706921|	*D. melanogaster*	5868
Catalase Gmm-2969	gi|24666457|	*D. melanogaster*	20810
Dual oxidase	gi|281364292|	*D. melanogaster*	4481
Glutathione peroxidase	gi|24656776|	*D. melanogaster*	4381, 7185, 3900
Glutathione S-transferase	gi|281361645|	*D. melanogaster*	441, 1341, 1174
Immune responsive catalase	gi|320544464|	*D. melanogaster*	5662
Isocitrate dehydrogenase	gi|24660859|	*D. melanogaster*	1163,2406
Malate dehydrogenase	gi|24583393|	*D. melanogaster*	589,700
Nitric oxide synthase	gi|6707649|	*D. melanogaster*	11319, 4149
Oxidation resistance 1	gi|221377882|	*D. melanogaster*	2088,4393,5202,9456
Peroxiredoxin Gmm-0929	gi|281362004|	*D. melanogaster*	4079
Peroxiredoxin Gmm-2087	gi|320544464|	*D. melanogaster*	2969,1235
Superoxide dismutase Gmm-1566	gi|195431735|	*D. willistoni*	1186
Superoxide dismutase Gmm-2338	gi|116007680|	*D. melanogaster*	2559,2079
Thioredoxin reductase	gi|24640552|	*D. melanogaster*	1857

5.2.4 Gene sequence extensions using genome sequence: gmorsitans.CONTIGS-Yale.GmorY1.fa

Corresponding nucleotide contig sequences generated from BLAST searches for NOS, Duox and OXR1 were translated into 6 predicted protein sequences, three forward and three reverse/complimentary frames (http://www.vivo.colostate.edu/molkit/translate/index.html). Protein sequences of the *Drosophila* homologs of NOS, Duox and OXR1 were then used to assemble and extend existing protein sequences of *Glossina's* NOS, Duox and OXR1. Fuller length extended protein sequences were then aligned and viewed to the corresponding *Drosophila* homologs' protein sequences using ClustalW. After the extended protein sequences were verified, the nucleotide sequences were assembled using text output of amino acids and DNA. Nucleotide sequences were verified using BLASTn and BLASTx and protein sequences using BLASTp.

5.2.5 Domain checks for predicted proteins

In order to confirm that conserved domains are present for each ORF, the amino acid sequences for NOS, Duox and OXR1 were analyzed using the conserved domain database (CDD) from Entrez (Marchler-Bauer *et al.* 2009). The regions of each domain of interest and the key amino acid residues for each domain were then noted.

5.3 Results

5.3.1 Phylogenetic analysis

Using representative NOS sequences from other organisms, a phylogenetic tree was generated using 1000 bootstrap replicates using the neighbour-joining algorithm. The *G. m. morsitans* NOS GMsg-7530 (GeneDB accession number GMsg-7530) selected from the salivary gland EST library (Alves-Silva *et al.* 2010) appears to be truncated form of the putative tsetse NOS gene, NCBI ADD18407.1 (Figure 5.1 Panel A). An 8.7% difference in homology between the two putative NOS genes is observed, due to the changes in the last 25 amino acids at the C-terminus of NOS GMsg-7530 compared to NOS ADD18407.1 which is longer. A radial phylogram was drawn from protein sequences of inducible NOS (iNOS) from *H. sapiens* and *R. norvegicus* and invertebrate NOS. The tsetse NOS GMsg-7530 deduced amino acid sequence showed greatest amino acid similarity (97.7%) with *Drosophila* and revealed a high level of homology with other members of the Diptera (Figure 5.1 Panel B). This is expected since all the dipterans should have a common ancestor, and therefore are related to each other. The subspecies of *Drosophila* and anophelines also cluster together since they contain high sequence homology. *M. sexta, P. humanus, Ap. mellifera* and *I. scapularis* are all very distantly related to *Glossina,* and *H. sapiens* and *R. norvegicus* are outliers as expected.

A phylogenetic tree using the neighbour-joining algorithm with 1000 bootstrap replicates was also constructed for Duox (Figure 5.2 Panel A). The *Glossina* Duox is closely related to dipterans, particularly to *Drosophila* (91.3% homology) while being distantly related to the mosquitoes *An. gambiae, Cx. quinquefasciatus* and *Ae. aegypti.* The *Drosophila* subspecies cluster together and so do the mosquitoes since they have high sequence homology. *H. sapiens* and *R. norvegicus* are outliers again. Figure 5.2 Panel B shows a radial phylogram of *Glossina* OXR1 closely related to *Drosophila* OXR1 (63.9% homology), with the same expected outliers.

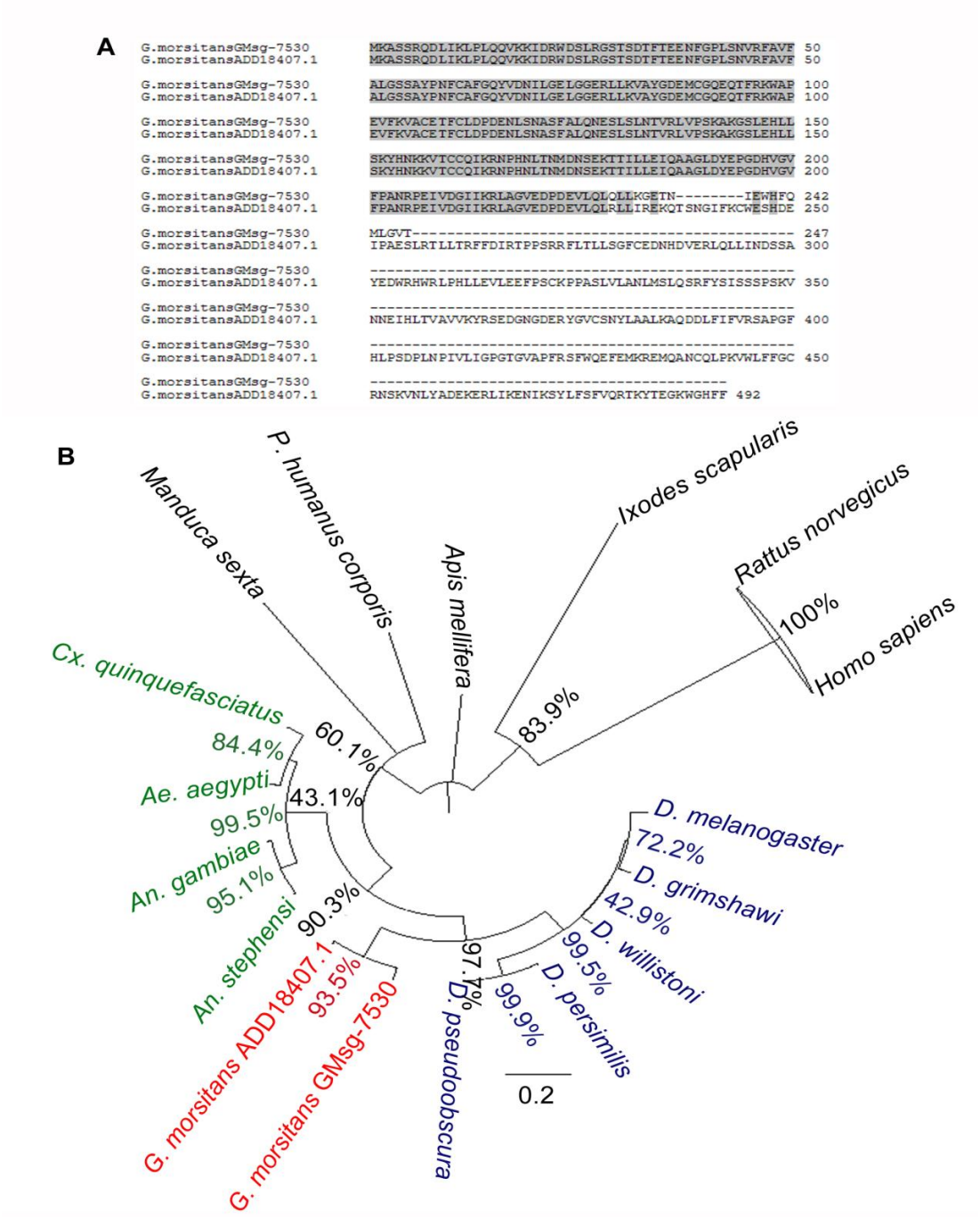


Figure 5.1. Alignment of *G. m. morsitans* NOS genes and maximum likelihood phylogenetic analysis of nitric oxide synthase (NOS) based on amino acid sequences of *G. m. morsitans* and other organisms including insects and human. (A) Alignment of the two protein sequences of *G. m. morsitans* NOS genes, GMsg-7530 and ADD18407.1. Identical sequences are highlighted in grey. **(B)** The radial phylogram was produced by neighbour-joining criteria with 1000 bootstrap replicates. Numbers at branches (%) correspond to bootstrap values. Outliers are the iNOS group: *Homo sapiens* and *Rattus norvegicus*. Invertebrate NOS includes: *Glossina* (red), *Drosophila* (blue), mosquitoes (green) and the remaining invertebrates (black).

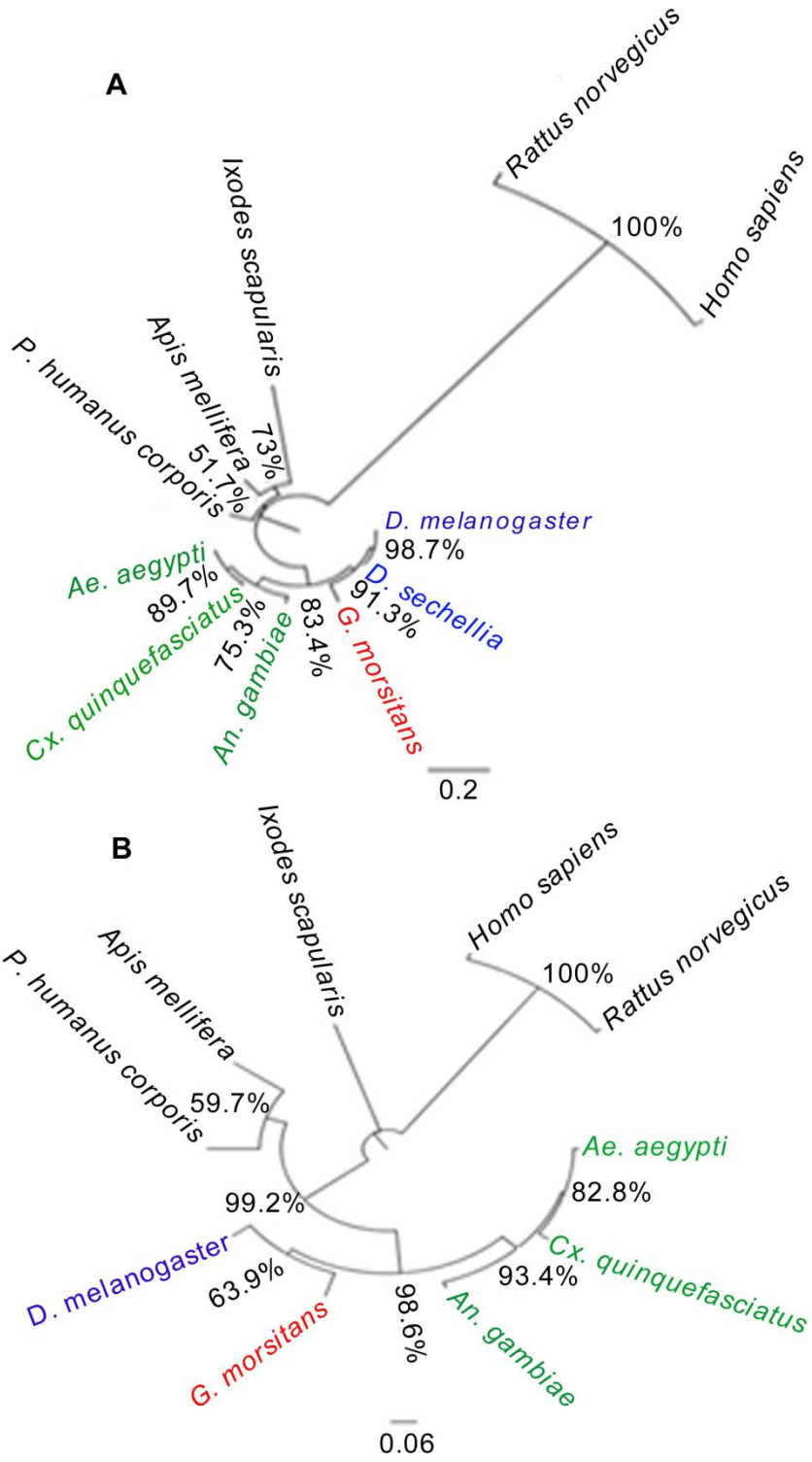


Figure 5.2. Maximum likelihood phylogenetic analysis of dual oxidase (Duox) and oxidation resistance 1 (OXR1) based on amino acid sequences of *G. m. morsitans* and other organisms including insects and human. (A) The radial phylogram of Duox was produced by neighbour-joining criteria with 1000 bootstrap replicates. Numbers at branches (%) correspond to bootstrap values. **(B)** Radial phylogram of OXR1.

5.3.2 Differential gene expression between midgut libraries using 454 data

Using the edgeR package, a tagwise dispersion analysis (by Alistair Darby) revealed 184 differentially expressed genes ($P < 0.05$) between the two midgut libraries (flies fed normal blood and flies fed trypanosome-infected blood) (Figure 5.3). Top-ranking genes (highest fold-change) from the set of 184 were then narrowed down to 70 for down and up regulated genes. Biological process annotation of down and up regulated genes were identified using BLAST2GO (Conesa *et al.* 2005) and are shown in Figure 5.4 Panels A and B respectively. In flies fed trypanosomes, down regulated genes included 22% of genes involved in cellular processes (cell cycle and transmembrane transport proteins), 17% involved in metabolic processes (oxidation-reduction, biosynthetic and lipid metabolism), 9% in response to stimulus (transferrin, carbon-nitrogen hydrolase) and 2% in immune system processes. Other genes found down regulated but not identified using biological process ontology included serine protease inhibitors and chitinases (Table 5.3). In the same set of flies challenged with trypanosomes, up regulated genes included 16% of metabolic process genes, 16% of cellular process genes, 5% of genes involved in cell death, 14% of genes in response to stimulus and 8% of genes involved in signalling. Genes found up regulated include heat-shock proteins and serine proteases (Table 5.4). NOS, Duox and OXR1 are present in the data set but not differentially expressed between the two data sets. The antioxidant gene list from Table 5.2 was searched in the data set to check for differential expression, however none were found to be differentially expressed.

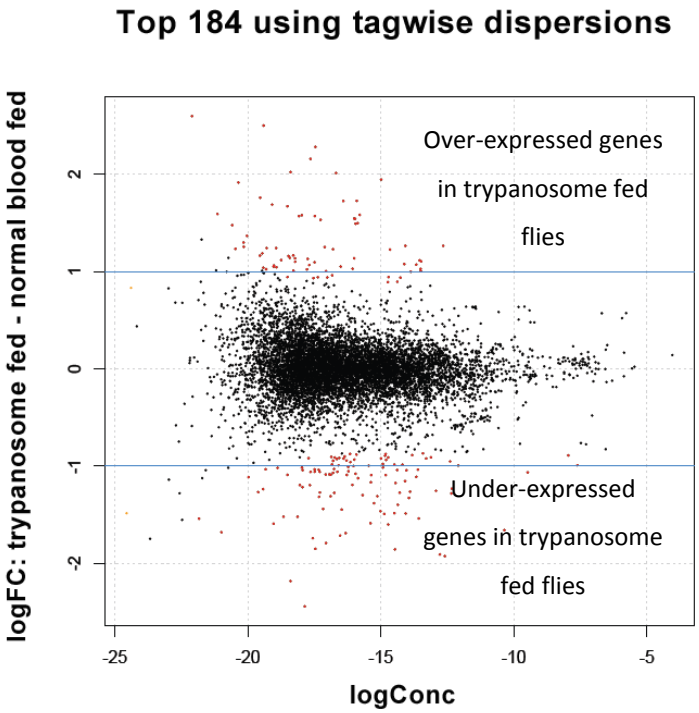


Figure 5.3. Plot of the log-fold change against the log-concentration for each tag. The 184 most differentially expressed tags (P<0.05) between the two midgut libraries (trypanosome fed and normal blood fed) using the tagwise dispersion (edgeR package) are outlined in red. (Adapted from source obtained from Alistair Darby, unpublished results).

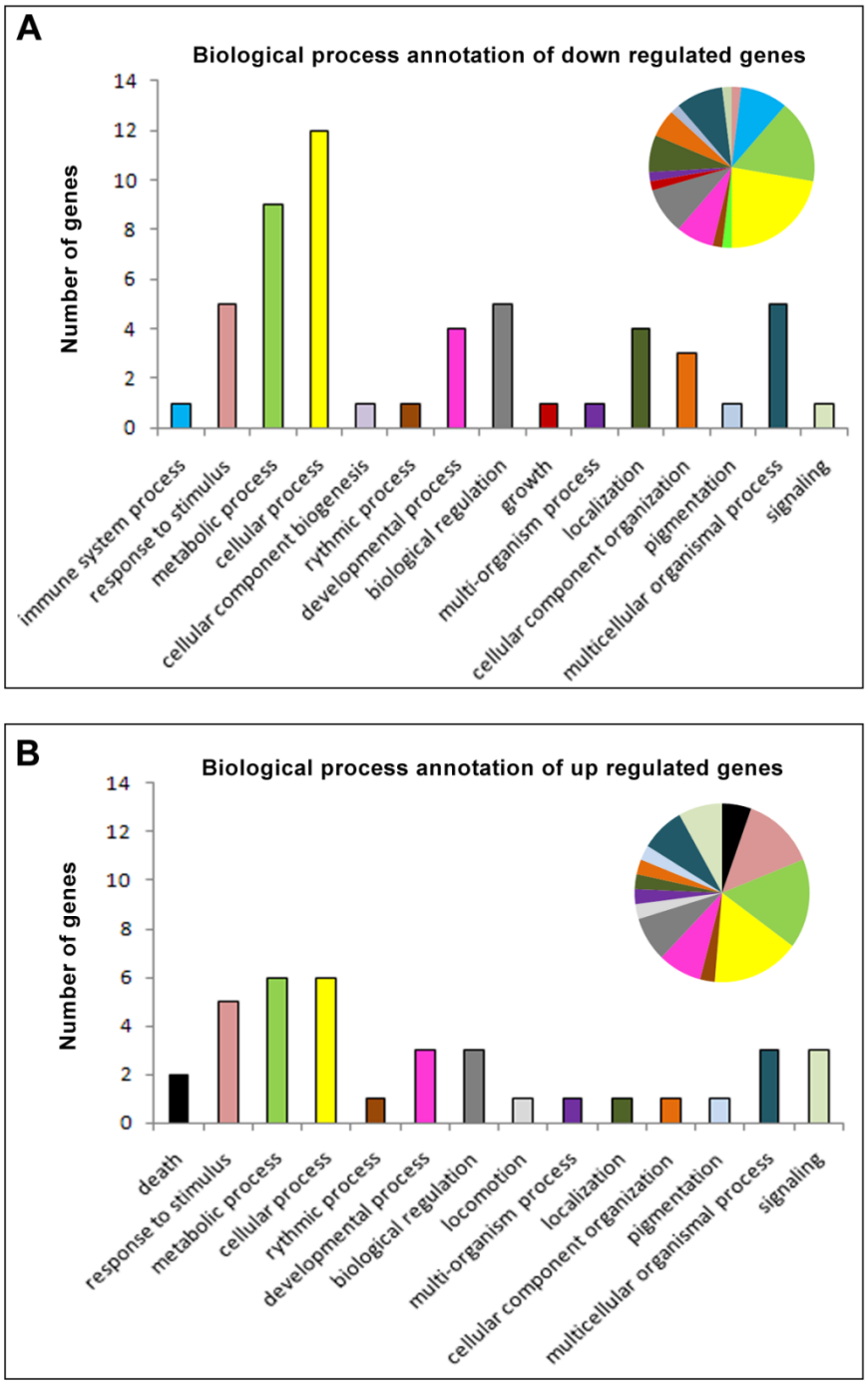


Figure 5.4. Classification of differential expressed genes using BLAST2GO. The 'biological process' ontology was used to map broad level terms to summarize individual annotations. **(A)** Annotation of down regulated genes in flies fed trypanosomes. **(B)** Annotation of up regulated genes in flies fed trypanosomes. Inserts: pie charts summarize the distribution of the GO terms (biological processes) of the genes.

Table 5.3. List of genes that are under-expressed (P<0.05) in trypanosome fed flies. Accession numbers and putative functions are listed. Table lists genes according to descending order of fold change (highest fold change at the top).

Accession numbers	Putative function	Comments
gi|289740011|	Homogentisate dioxygenase	
gi|289741725|	Synaptic vesicle protein	
gi|198474814|	Calcium channel- exocytosis	
gi|195118445|	Slc26 membrane transporter protein	High affinity sulphate transmembrane transporter activitiy
gi|289739719|	Hypothetical conserved protein	Unknown
gi|194755693|	GF13208 *D. ananassae*	Unknown
gi|195427359|	GK17163 *D. willistoni*	Unknown
gi|195144790|	GL24111	Unknown
gi|289743255|	Transferrin precursor	Immune related
gi|195149327|	Secretory phospholipase isoform a	Antibacterial
gi|289740065|	Serine proteinase inhibitor	Immune related
gi|18201665|	Chitinase 4	Immunity related Verleyena Drosophila
gi|289740709|	Aquaporin	
gi|195483598|	Isoform b	Glyoxylate and dicarboxylate metabolism
gi|289742415|	Hypothetical conserved protein	Unknown
gi|289742413|	Hypothetical conserved protein	Unknown
gi|289743255|	Transferrin	Immune related
gi|289742407|	Hypothetical conserved protein	Unknown
gi|21311570|	Chymotrypsin inhibitor-like	Immune reactive putative protease inhibitor
gi|195390598|	James isoform a	Meiosis - cytokinesis
gi|195398554|	Alcohol dehydrogenase	Oxidation-reduction
gi|195433519|	CG15293	Unknown

gi|289743353|	Carbon-nitrogen hydrolase	Heat tolerance and JNK sensitivity
gi|289743015|	Hypothetical conserved protein	Unknown
gi|289741973|	Monocarboxylate transporter	Transport processes
gi|195434128|	U6 snrna-associatiated sm-like protein lsm4	
gi|289724671|	M13 family peptidase	Metalloendopeptidase
gi|289724540|	Mucin related 18b/ glycoprotein	
gi|289742187|	Acid sphingomyelinase	
gi|289739719|	Hypothetical conserved protein	Unknown
gi|289740075|	Glycogenin	Glucose to glycogen
gi|289743353|	Carbon-nitrogen hydrolase	
gi|289741287|	Mitochondrial ribosomal protein	Alpha glucosidase
gi|289724540|	Glycoprotein	
gi|195400118|	Tpa_inf:hdc14388	Unknown
gi|289743031|	Gmfb8d	Unknown
gi|83944670|	Retinoid & fatty acid-binding glycoprotein/ lipophorin	
gi|289739835|	Isoform a /hypothetical conserved protein	Hemolymph coagulation - Toll regulated protein in *Drosophila*
gi|289740385|	Serine protease inhibitor 43ab	

Table 5.4. List of genes that are over-expressed (P<0.05) in trypanosome fed flies. Accession numbers and putative functions are listed. Table lists genes according to descending order of fold change (highest fold change at the top).

Accession numbers	Putative function	Comments
gi|195112224|	Unknown SEL 1L	Plasma membrane, binding
gi|195053596|	Isoform a; zinc ion binding, nucleic acid binding	
gi|195430888|	Acetyl glucosaminyltransferase activity - protein amino acid glycosylation	Super sex isoform b; wing development, protein amino acid glycosylation, acetyl glucosaminyltransferase activity
gi|289744025|	Hypothetical secreted peptide precursor	
gi|289741783|	Heat shock protein cognate 4	
gi|289741003|	Sphingomyelin phosphodiesterase 1	
gi|289741053|	F-box protein; Unknown	Hydrolase activity
gi|289741499|	60s acidic ribosomal protein P1	Translational elongation
gi|195582909|	Protein binding - autophagy - translation initiation factor	Eukaryotic translation initiation factor 4; N-acetyltransferase activity
gi|195117928|	mRNA binding - negative regulation of translation	
gi|289740037|	Smell impaired 21F	
gi|195427359|	GK17163; inter-male aggressive behaviour	
gi|289741437|	Zn finger protein	Zinc ion binding
gi|194752443|	Serine-type endopeptidase activity	
gi|289743671|	Farnesoic acid O-methyltransferase; Juvenile hormone biosynthetic pathway. Juvenile hormone immunosuppressor.	
gi|289743395|	Decondensation factor 31; decondensation of chromatin	
gi|198470190|	GL13366	Unknown

5.3.3 Gene sequence extension using 454 data

Antioxidant genes (from Table 5.2) from *Drosophila* were selected from FlyBase and BLAST searches were performed using stand alone BLAST of *Drosophila* homologs against the midgut cDNA data to obtain corresponding contigs. Nucleotide and protein sequences of corresponding contigs were aligned to existing *Glossina* sequences and orthologs from other dipterans. However, the protein sequences of *Glossina* genes were almost similar in length to other dipterans and contigs retrieved from midgut cDNA 454 data also were roughly of the same length, therefore no extension was needed. Table 5.5 shows an example of an alignment of the protein sequences of glutathione S-transferase of *Glossina*, contigs retrieved from the 454 data set and other dipterans.

Table 5.5. Alignment of protein sequences of existing glutathione S-transferase gene of *G. m. morsitans* (red) aligned against contigs from 454 data and other dipterans.

Sequence comparison

```
Contig441/1-208          ------------------------MDLYYLPGSAPCRSVIMTAKALGLQLNKKLLNLMAG 36
GmmADD20585.1|/1-208     ------------------------MDLYYLPGSAPCRSVIMTAKALGLQLNKKLLNLMAG 36
Contig1174/1-100         ------------------------MDLYYLPGSAPCRSVIMTAKALGLQLNKKLLNLMAG 36
Contig1341/1-99          ------------------------MDLYYLPGSAPCRSVIMTAKALGLQLNKKLLNLMAG 36
Drosophila/1-232         EFSATFHLIQCTSRVSQQKKKIKMVDFYYLPGSSPCRSVIMTAKAVGVELNKKLLNLQAG 60
Culex/1-207              ------------------------MDFYYLPGSAPCRAVQMTAAAVGVELNLKLTNLMAG 36
Anopheles/1-209          ------------------------MDFYYLPGSAPCRAVQMTAAAVGVELNLKLTDLMKG 36

Contig441/1-208          EQMKPEFLKLNPQHTIPTLVDGDFSIWESRAIMVYLVEKYGKTDSLFPKCPKKRAIINQR 96
GmmADD20585.1|/1-208     EQMKPEFLKLNPQHTIPTLVDGDFSIWESRAIMVYLVEKYGKTDSLFPKCPKKRAIINQR 96
Contig1174/1-100         EQMKPEFLKLNPQHTIPTLVDGDFSIWESRAIMVYLVEKYGKTDSLFPKMYP-----KKT 91
Contig1341/1-99          EQMKPEFLKLNPQHTIPTLVDGDFSIWESRAIMVYLVEKYGKTDSLFSQMS------QET 90
Drosophila/1-232         EHLKPEFLKINPQHTIPTLVDNGFALWESRAIQVYLVEKYGKTDSLYPKCPKKRAVINQR 120
Culex/1-207              EHMKPEFLKLNPQHCIPTLVDGSFPVWESRAIMIYLVEKYGKDESLYPKDPQKRAVVNQR 96
Anopheles/1-209          EHMKPEFLKLNPQHCIPTLVDNGFALWESRAIQIYLAEKYGKDDKLYPKDPQKRAVVNQR 96

Contig441/1-208          LYFDMGTLYKSFADYYYPQIFSKAPADPEMHKKIETAFDFLNTFLEGQQYAAGDTLTVAD 156
GmmADD20585.1|/1-208     LYFDMGTLYKSFADYYYPQIFSKAPADPEMHKKIETAFDFLNTFLEGQQYAAGDTLTVAD 156
Contig1174/1-100         GYYQSASIL---------------------------------------------------- 100
Contig1341/1-99          GYYQSASIL---------------------------------------------------- 99
Drosophila/1-232         LYFDMGTLYQSFANYYYPQVFAKAPADPEAFKKIEAAFEFLNTFLEGQDYAAGDSLTVAD 180
Culex/1-207              LFFDQGTLYQRFADYFYPQIFAKQPANADNEKKMLDGLDFLNTFLGGSKYVAGDQLTIAD 156
Anopheles/1-209          LYFDMGTLYQRFADYHYPQIFAKQPANPENEKKMKDAVGFLNTFLEGQEYAAGNDLTIAD 156

Contig441/1-208          IALLATVSTFEVAGFDFSKYPNVAKWYANAKTVTPGFDENWQGCLEFKKFFN- 208
GmmADD20585.1|/1-208     IALLATVSTFEVAGFDFSKYPNVAKWYANAKTVTPGFDENWQGCLEFKKFFN- 208
Contig1174/1-100         ------------------------------------------------------
Contig1341/1-99          ------------------------------------------------------
Drosophila/1-232         IALVATVSTFEVAKFEISKYANVNRWYENAKKVTPGWEENWAGCLEFKKYFE- 232
Culex/1-207              LTILATVSTYDVAKVDLAKYPNVAGWYARLRKEAPGAAINEAGCEEFKKYF-- 207
Anopheles/1-209          LSLAATIATYEVAGFDFAPYPNVAAWFARCKANAPGYALNQAGADEFKAKFLS 209
```

5.3.4 Gene sequence extension and domain checks using available genome sequence

Nitric oxide synthase (NOS): The original *G. m. morsitans* NOS sequences (GMsg-7530 and ADD18407.1) contained only the reductase domain of NOS. Stand alone BLAST searches using tBLASTx and BLASTn of NOS nucleotide sequence retrieved Gap.contig.14987 from the *Glossina* genome contigs (gmorsitans.CONTIGS-Yale.GmorY1.fa). Translation of contig 14987 resulted in 6 predicted protein sequences, three in the forward frames and three in the reverse/complimentary frames. Using *Drosophila* NOS sequence as a template, the protein sequence of *Glossina* was extended further at both the N and C terminus by assembling the protein sequences from the different reading frames. Figure 5.5 shows a schematic representation of the assembly process of all three genes. Conserved domains that are crucial to gene functions were searched using the extended protein sequences of NOS, Duox and OXR1 with CDD.

The extended NOS protein sequence is 1,157 amino acids (Table 5.6) and the nucleotide sequence being 3,471 bp (Appendix II). The original protein sequence (GMsg-7530) was 247 amino acids. The new protein sequence has been extended by 79% at both the N and C terminus and comparable in size to *Drosophila* NOS at 1,244 amino acids. NOS consists of two similar monomers which can be divided into 2 major domains including a C-terminal reductase domain (residues 844-1,157) which binds to NADPH, FAD and FMN and an N-terminus oxygenase domain (residues 244-527) which binds to haem, BH_4 and the L-Arginine substrate. The original protein sequence covered only the reductase domain, however with the new extended sequence, the oxygenase domain has now been detected together with other smaller domains including FAD binding (residues 829-1,056) (Table 5.7).

Dual oxidase (Duox): The original *G. m. morsitans* Duox sequence of 223 amino acids in full length (GMre-16d11.q1k) contained only the calcium binding domain. Stand alond BLAST searches using tBLASTx and BLASTn of Duox nucleotide sequence retrieved Gap.contig.47345 from the *Glossina* genome contigs (gmorsitans.CONTIGS-Yale.GmorY1.fa). Using *Drosophila* Duox sequence as a template, the protein sequence of *Glossina* was extended further at both the N and C terminus by assembling the protein sequences from the different reading frames. The extended Duox protein sequence is 1,215 amino acids (Table 5.6) and the nucleotide sequence being 3,645 bp (Appendix II). The original protein sequence (GMre-16d11.q1k) was 223 amino acids. The new protein sequence has been extended by 82% at both the N and C terminus and comparable in size

to *Drosophila* Duox at 1,475 amino acids. The new extended Duox protein sequence now contains the major domain needed for its function in immunity, the dual peroxidase domain (residues 1-538 in Table 5.7). It also contains the FAD-binding (residues 1,175-1,215) and ferric reductase domains (residues 997-1115).

Oxidation resistance 1 (OXR1): The original *G. m. morsitans* OXR1 sequence of 297 amino acids in full length (Gmm-2826) contained the major domain TLDc. Stand alone BLAST searches using tBLASTx and BLASTn of Duox nucleotide sequence retrieved Gap.contig.47345 from the *Glossina* genome contigs (gmorsitans.CONTIGS-Yale.GmorY1.fa). Using *Drosophila* OXR1 sequence as a template, the protein sequence of *Glossina* was extended further at the N terminus by 433 amino acids to make a fuller length sequence of 730 amino acids (Table 5.6). The extended sequence now represents the Lysin domain as well (Table 5.7) in addition to the TLDc domain.

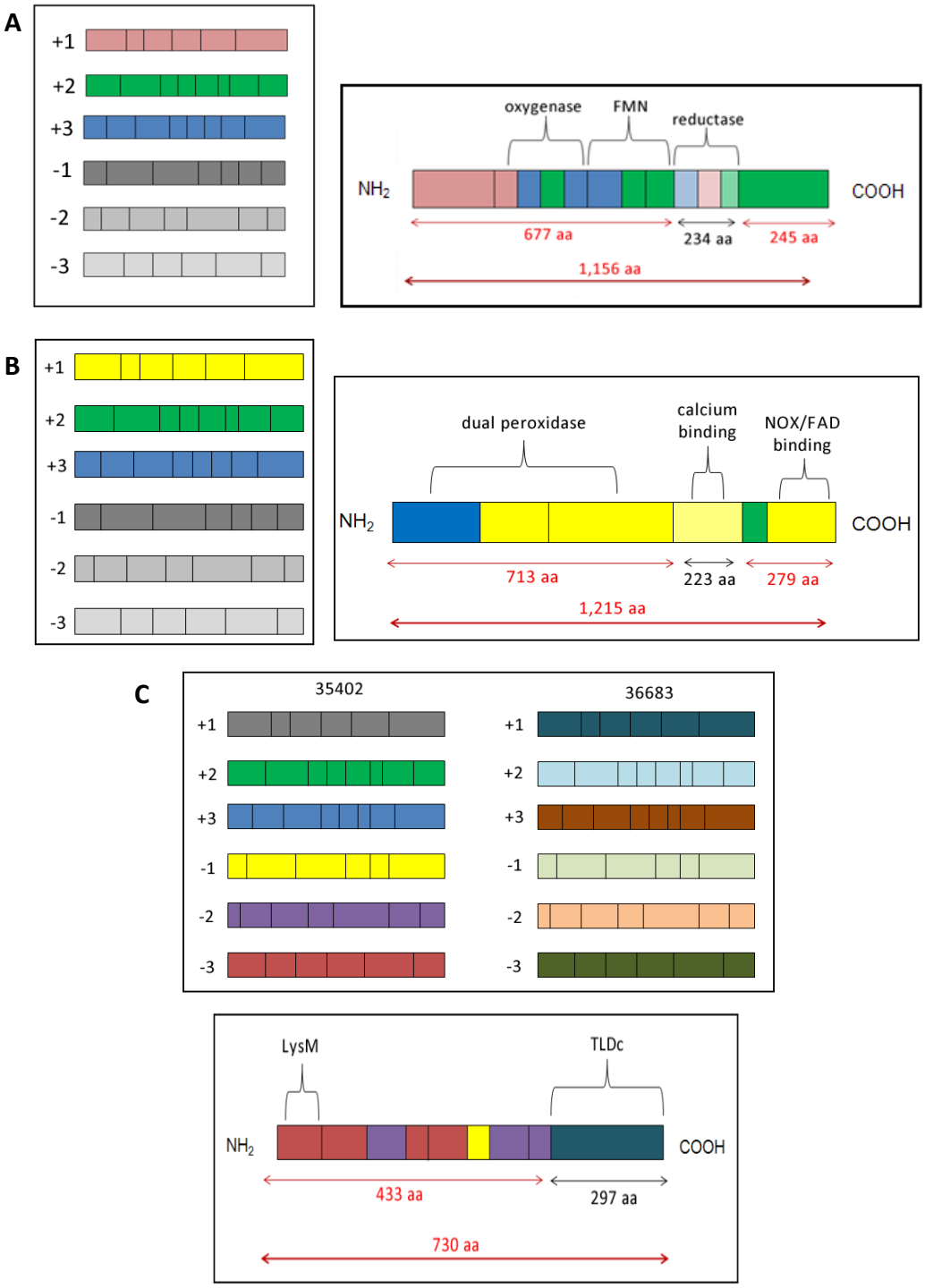


Figure 5.5. Schematic representation of NOS, Duox and OXR1 reading frames and extended protein sequence. +1, +2, +3, -1, -2, -3 represent the different reading frames; the extended sequences were assembled using exons from the different frames. Red arrows represent extended sequences in amino acids (aa). **(A)** NOS extended protein sequence **(B)** Duox extended protein sequence **(C)** OXR1 extended protein sequence from 2 contigs: 35402 and 36683. Extended amino acids in red.

Table 5.6. Extended protein sequences of nitric oxide synthase (NOS), dual oxidase (Duox) and oxidation resistance 1 (OXR1) and their sizes. Highlighted in grey are the extended sequences and in white are the original sequences before extension.

Name	Sequence	Size
NOS protein	MSHFTSILENIRLVAIKRSPLSQQKQSNKEQQSSTSHPPSNTATITITDTDLLQQQQADIHNEYNKSSKVLNTTTSARITTSKTISSNAHAYQHEDQADKGEIANNSHISGNVAT NAISGTSVKAHSKRQSVSARGSISSGENVRTSPGRERNVSNRTANLIAEIDINGSNSSVSGVKNSVTRTRDLSPSPKNQQRRMSTDFRARAGSFIHVDDEGRSILMRKPVRLK NIEGRPEVYDTLHFKGFQILSCSKSTCMSSVMNFGTAPVEARKSEVVLEHAKDFLDQYFTSIKRSSSAAHEARWKQVRQSIESTGHYQLTETELIYGAKLAWRNSSRCIGRIQ WSKLQVFDCRYVTTTSGMFEAICNHIKYATNKGNLRRSAITIFPQRTDARHDYRIWNTQLISYAGYKQNDGKIIGDPLNVEFTTVALKMNLDTRTPTSLWKDKAVVEVNIAVL YSYQSRNVTIVDHHTASESFMKHFENESKLRNGCPADWIWIVPPLSGSITPVFHQEMALYYLKPSFEYQDPAWRTHIWKKGRGDGKSKKPRRKFNFKQIARAVKFTSKLFGR ALSKRIKATVLYATETGKSEKYAKQLCELLGHAFNAQIYCMSEYDISSIEHEALLIVVASTFGNGDPPENGELFSVCVCLIFFFSNICENLIDKISFKCFFSIGVTSSKSFMKASSRQD LIKLPLQQVKKIDRWDSLRGSTSDTFTEENFGPLSNVRFAVFALGSSAYPNFCAFGQYVDNILGELGGERLLKVAYGDEMCGQEQTFRKWAPEVFKVACETFCLDPDENLSN ASFALQNESLSLNTVRLVPSKAKGSLEHLLSKYHNKKVTCCQIKRNPHNLTNMDNSEKTTILLEIQAAGLDYEPGDHVGVFPANRPEIVDGIIKRLVGVEDPDEVLQLQLLKEK QTSNGIFKCWESHDKIPAESLRTLLTRFFDITTPPSRQFLTLLSGFCEDNHDVERLQLLINDSSAYEDWRHWRLPHLLEVLEEFPSCKPPASLVLANLMSLQSRFYSISSSPSKVN NEIHLTVAVVKYRSEDGNGDERYGVCSNYLAALKAQDDLFIFVRSAPGFHLPSDPLNPIVLIGPGTGVAPFRSFWQEFEMKREMQANCQLPKVWLFFGCRNSKVNLYADEK ERLIKENILNRIFLALSRE	1,157 aa
Duox protein	SHLVRKAPPSYSDGVYALAGLNRPSTRRLSRLFMRGRDGLGSKYNRTALLAFFGQVVANEIVMASESGCPIEMHRIEIEKCDEMYDKACRGDKYIPFHRAAYDRNTGSSPNA PREQINQMSAWIDGSFIYSTSEAWLNAMRSFHNGSLLTDKSGKLPIRNTMRVPLFNNPVPNVMKMLSPERLFAVLGDPRTNQNPAVLSFAILFLRWHNTWAQRVKQAHP GWSDEDIFQKARRYVIASLQNVIMYEYLPAFLASEIPPYEGYKQDIHPGVGHIFQAAAFRFGHTMIPPGIYRRDANCNYKATAMGYPAIRLCSTWWDSSDFLADTKVEEILM GLSSQISEREDPVLCSDVRDKLFGPMEFTRRDLGALNIMRGRDNGLPDYNSARVAFGLLEHNNWTDINPDLFSKQPELLESLISAYNNRLDDVDVYVGGMLESYGQPGELFS TVIKEQFQRLRDADRFWFENDKNGIFAPEEIEEIRKIRMYDIIVNSTDINPQHIQKNVFIWHKGDPCPQPMQLNATELEPCTHLEGYDYFSGSELMFIYVCVFLGFVPILCAGA SYCVVKLQNSKRRKLKIRQEALRSPQHKGSVDKMLAREWLHANHKRLVTVKFGPEAAIYTVDRKGEKLRTFSLKNIDLVTVEESATNHIKKKPYILLRVPNDHDLVLELESYGA RRKFVKKLEDFLILHKKEMTLVEVNRDIMLARAETRERRQKRLEYFFREAYALTFGLRPGERRRRSDASTDGEVMTVMRTSLSKAEFAAALGMKPNDMFVRKMFNIVDKDQ DSRISFQEFLETVVLFSRGKTDDKLRIIFDMCDNDRNGVIDKGELSEMMRSLVEIARTTSLGDDQVTELIDGMFQDVGLEHKNHLTYQDFKLMMKEYKGDFVAIGLDCKGAK QNFLDTSTNVARMTSFNIEPMQERPRYWMEVKWDSYITFLEENRQNIFYLFLFYVITIVLFVERFIDYSFMAEHTDLRHIMGVGIAITRGSAASLSFCYSLLLLTMSRNLITKLKE FPIQQYIPLDSHIQFHKIAACTALFFSVLHTVGHIVNFYHVSTQSHENLRCLTREVHFASDYKPDITFWLFQTVTGVLLFIIMCIIFVFAHPTIRKKAYTFFWNMHSLYIALYLLSLI HGLARLTGPPRFWMFFLGPGVIYTLDKIVSLRTKYMALDVIETELLPSDVIKIKFYRPPNLKYLSGQWVRLSCTAFQPHEMHSFT	1,215 aa
OXR1 protein	KSSLPTIPTINYTVGNRDTLTSVAARFDTTPSELTHLNRLNSSFIYPGQQLLVPDKSAKDDILESLRPGSPKPGRIERIDVNDRSDMNKTDDPIITQRFLKINVRHITDGQGVVGG VLLVTPNAVMFDPNVSDPLVIEHGPESYGVIAPMELCVNAAIFHDIAHMRVSGGAVISADNTNATEKPEIYYPKAVLEATLEERRKSLLDHHWAIPSKDRLEQLEQLTKQSCY DSGIDIREPIPNVQPIPKKTVYSDADIVLSSDWVPPKPISTTPLSESPPRSSGMATCQSLDAGTGGTRKKTSSVSFSVDDMLKRLSYPLTWVEGLTGDATSSAVAGVSKSIDTDS APNTGDSNQSVFSKVFSRRSSIGTFMRPTSSEGSSSAIKTKEVKVQPKLDYRSMVSVDDKPELFVSVDSKLIPRPARACPDPPLYLRLRMGKPTGKTIPLPTSVMSYGKNKLRP EYWFSVPKNRVDELYRFMNTWVPHLYGELDEEQIKNRGFELIQDDTEWTQSGTKKSGSRSNSQEGEEISDLTRESWEVLSMSNDDYRKASIFQTGSFDLDFPIPELIGTTEILT EEHREKLCSHLPARAEGYSWSLVFSTSLHGFSLNSLYRKMQRLESPILIVIEDTDHNVFGALTSCSLHVSDHFYGNGESLLYKFNPSFKVFHWTGENLYFIKGNVESLLVGAGNG RFGLWLDGDLNQGRSQSCSTYGNEPLAPQEDFVIKTLEGWAFV	730 aa

Table 5.7. The extended protein sequences of tsetse nitric oxide synthase (NOS), dual oxidase (Duox) and oxidation resistance 1 (OXR1) and their conserved domain hits on CDD. Different domains are highlighted in different colours and characteristic conserved domain residues are highlighted in red. The original protein sequence pre-extension is underlined.

Domain type	Extended protein sequences	Domain residues	Conserved domain residues
NOS NOS oxygenase FMN – reductase FAD binding **Ferroxiredoxin-reductase (FNR)**	MSHFTSILENIRLVAIKRSPLSQQKQSNKEQQSSTSHPPSNTATITITDTDLLQQQQADIHNEYNKSSKVLNTTTSARITTSKTISSNAHAYQHED QADKGEIANNSHISGNVATNAISGTSVKAHSKRQSVSARGSISSGENVRTSPGRERNVSNRTANLIAEIDINGSNSSVSGVKNSVTRTRDLSPSP KNQQRRMSTDFRARAGSFIHVDDEGRSILMRK**PVRLKNIEGRPEVYDTLHFKGFQILSCSKSTCMSSVMNFGTAPVEARKSEVVLEHAKDF** **LDQYFTSIKRSSSAAHEARWKQVRQSIESTGHYQLTETELIYGAKLAWRNSSRCIGRIQWSKLQVFDCRYVTTTSGMFEAICNHIKYATNKG** **NLRRSAITIFPQRTDARHDYRIWNTQLISYAGYKQNDGKIIGDPLNVEFTTVALKMNLDTRTPTSLWKDKAVVEVNIAVLYSYQSRNVTIV** **DHHTASESFMKHFENESKLRNGCPADWIWIVPPLSGSITPVFHQEMALYYLKPSFEYQDPAW**RTHIWKKGRGDGKSKKPRRKFNFKQIAR AVKFTSKLFGRALSKRIKATVLYATETGKSEKYAKQLCELLGHAFNAQIYCMSEYDISSIEHEALLIVVASTFGNGDPPENGELFSVCVCLIFFFSNI CENLIDKISFKCFFSIGVTSSKSFMKASSRQDLIKLPLQQVKKIDRWDSLRGSTSDTFTEENFGPLSNVRFAVFALGSSAYPNFCAFGQYVDNILG ELGGERLLKVAYGDEMCGQEQTFRKWAPEVFKVACETFCLDPDENLSNASFALQNESLSLNTVRLVPSKAKGSLEHLLSKYHNKKVTCCQIKR **NPHNLTNMDNSEKTTILLEIQAAGLDYEPGDHVGVFPANRPEIVDGIIKRLVGVEDPDEVLQLQLLKEKQTSNGIFKCWESHDKIPAESLRT** **LLTRFFDITTPPSRQFLTLLSGFCEDNHDVERLQLLINDSSAYEDWRHWRLPHLLEVLEEFPSCKPPASLVLANLMSLQSRFYSISSSPSKVNN** **EIHLTVAVVKYRSEDGNGDERYGVCSNY**LAALKAQDDLFIFVRSAPGFHLPSDPLNPIVLIGPGTGVAPFRSFWQEFEMKREMQANCQLPKV WLFFGCRNSKVNLYADEKERLIKENILNRIFLALSRE	244-527 **844-1157**	357, 421 874
		576-640 709-775 829-1056	- -

Domain type	Extended protein sequences	Domain residues	Conserved domain residues
Duox Dual peroxidase	SHLVRKAPPSYSDGVYALAGLNRPSTRRLSRLFMRGRDGLGSKYNRTALLAFFGQVVANEIVMASESGCPIEMHRIEIEKCDEMYDKACRGDK YIPFHRAAYDRNTGSSPNAPREQINQMSAWIDGSFIYSTSEAWLNAMRSFHNGSLLTDKSGKLPIRNTMRVPLFNNPVPNVMKMLSPERLF AVLGDPRTNQNPAVLSFAILFLRWHNTWAQRVKQAHPGWSDEDIFQKARRYVIASLQNVIMYEYLPAFLASEIPPYEGYKQDIHPGVGHIFQ AAAFRFGHTMIPPGIYRRDANCNYKATAMGYPAIRLCSTWWDSSDFLADTKVEEILMGLSSQISEREDPVLCSDVRDKLFGPMEFTRRDLGA LNIMRGRDNGLPDYNSARVAFGLLEHNNWTDINPDLFSKQPELLESLISAYNNRLDDVDVYVGGMLESYGQPGELFSTVIKEQFQRLRDADR FWFENDKNGIFAPEEIEEIRKIRMYDIIVNSTDINPQHIQKNVFIWHKGDPCPQPMQLNATELEPCTHLEGYDYFSGSELMFIYVCVFLGFVPIL CAGASYCVVKLQNSKRRKLKIRQEALRSPQHKGSVDKMLAREWLHANHKRLVTVKFGPEAAIYTVDRKGEKLRTFSLKNIDLVTVEESATNHIK KKPYILLRVPNDHDLVLELESYGARRKFVKKLEDFLILHKKEMTLVEVNRDIMLARAETRERRQKRLEYFFREAYALTFGLRPGERRRRSDASTD GEVMTVMRTSLSKAEFAAALGMKPNDMFVRKMFNIVDKDQDSRISFQEFLETVVLFSRGKTDDKLRIIFDMCDNDRNGVIDKGELSEMMRS LVEIARTTSLGDDQVTELIDGMFQDVGLEHKNHLTYQDFKLMMKEYKGDFVAIGLDCKGAKQNFLDTSTNVARMTSFNIEPMQERPRYWM EVKWDSYITFLEENRQNIFYLFLFYVITIVLFVERFIDYSFMAEHTDLRHIMGVGIAITRGSAASLSFCYSLLLLTMSRNLITKLKEFPIQQYIPLDSHI QFHKIAACTALFFSVLHTVGHIVNFYHVSTQSHENLRCLTREVHFASDYKPDITFWLFQTVTGVLLFIIMCIIFVFAHPTIRKKAYTFFWNMHSLY IALYLLSLIHGLARLTGPPRFWMFFLGPGVIYTLDKIVSLRTKYMALDVIETELLPSDVIKIKFYRPPNLKYLSGQWVRLSCTAFQPHEMHSFT	1-538 772-834 997-1115 1,175-1,215	194, 195, 277, 280-282, 284, 287, 316, 366, 369, 373 781, 783, 785, 792, 817, 819, 821, 828, 862, 864, 866, 873
OXR1 Lysin TLDc	KSSLPTIPTINYTVGNRDTLTSVAARFDTTPSELTHLNRLNSSFIYPGQQLLVPDKSAKDDILESLRPGSPKPGRIERIDVNDRSDMNKTDDPIITQ RFLKINVRHITDGQGVVGGVLLVTPNAVMFDPNVSDPLVIEHGPESYGVIAPMELCVNAAIFHDIAHMRVSGGAVISADNTNATEKPEIYYPK AVLEATLEERRKSLLDHHWAIPSKDRLEQLEQLTKQSCYDSGIDIREPIPNVQPIPKKTVYSDADIVLSSDWVPPKPISTTPLSESPPRSSGMATC QSLDAGTGGTRKKTSSVSFSVDDMLKRLSYPLTWVEGLTGDATSSAVAGVSKSIDTDSAPNTGDSNQSVFSKVFSRRSSIGTFMRPTSSEGSSS AIKTKEVKVQPKLDYRSMVSVDDKPELFVSVDSKLIPRPARACPDPPLYLRLRMGKPTGKTIPLPTSVMSYGKNKLRPEYWFSVPKNRVDELYR FMNTWVPHLYGELDEEQIKNRGFELIQDDTEWTQSGTKKSGSRSNSQEGEEISDLTRESWEVLSMSNDDYRKASIFQTGSFDLDFPIPELIGTT EILTEEHREKLCSHLPARAEGYSWSLVFSTSLHGFSLNSLYRKMQRLESPILIVIEDTDHNVFGALTSCSLHVSDHFYGNGESLLYKFNPSFKVFH WTGENLYFIKGNVESLLVGAGNGRFGLWLDGDLNQGRSQSCSTYGNEPLAPQEDFVIKTLEGWAFV	12-54 575-730	12, 15, 18, 23, 26

5.4 Discussion

5.4.1 Phylogenetic analysis

In response to pathogens and parasites, insects can mount an immune response which is made up of the ancient defence system of innate immunity (Christophides *et al.* 2002). This system encompasses a range of gene families with varying functions (physiological or developmental). Oxidative pathways form part of innate immunity and are particularly important in the insect gut (Ha *et al.* 2005a; Ha *et al.* 2005b; Lemaitre and Hoffmann 2007; Kumar *et al.* 2010) which we are particularly interested in. Consequently we used a phylogenetic approach to compare tsetse sequences of NOS, Duox and OXR1 to counterparts from other dipterans and vertebrates.

In vertebrates, there are three distinct NOS isoforms on separate chromosomes: two constitutively expressed forms, endothelial NOS (eNOS) and neuronal NOS (nNOS) and the third being an inducible NOS (iNOS) (Luckhart and Li 2001). It is not yet clear whether all three isoforms of NOS are present in insects, however only one NOS isoform has been identified in insects (Luckhart *et al.* 1998). The sequence homology and phylogenetic analysis confirm a high level of similarity between tsetse NOS and other invertebrate NOS. In particular the constructed radial phylogram shows the dipterans clustering together, with NOS protein sequences of tsetse and *Drosophila* being highly similar (97.7%). The tick *I. scapularis* is isolated from *Glossina* in the phylogram and the blood feeders are interspersed between nectar feeders suggesting that diet has not driven NOS sequence homology. Duox and OXR1 protein sequences both show highest similarities to *Drosophila* (91.3% and 63.9% respectively). *H. sapiens* and *R. norvegicus* are expected outliers in the phylogenetic analysis of the three genes since *H. sapiens* and *R. norvegicus* represent the vertebrates.

5.4.2 Differentially regulated genes from 454 data

Insects rely on constitutive and inducible defence strategies to combat different microbial infections (Hao *et al.* 2001). Once recognized by host receptors, pathogens can activate proteolytic cascades in the midgut and fat body, leading to melanisation, opsonisation and synthesis of antimicrobial peptides (Hao *et al.* 2001). The immune response of tsetse to trypanosome infections can lead to complete elimination of parasites in challenged flies (refractoriness) while in susceptible flies where parasites establish themselves, the tsetse immune responses may prevent the accumulation of high parasite numbers to prevent

harming the host (Hao *et al.* 2001). We identified multiple immune-related genes which were either down regulated or up regulated in tsetse flies challenged with trypanosomes using 454 data obtained from two midgut libraries (normal blood fed flies and flies fed trypanosomes). Since flies were fed infectious blood *(T. b. brucei*-infected) at the fourth bloodmeal and midguts were dissected at day 3 post-infection, it is very likely that >90% of these flies would be self-cured. Typically midgut infection prevalence of flies infected at fourth bloodmeal is less than 10% (Distelmans *et al.* 1982; Haines *et al.* 2010). Day 3 post-infection was chosen for dissection because that is when key events are taking place in the fly, leading to a loss of trypanosomes in a proportion of flies (Gibson and Bailey 2003). Thus we have the optimal chance of seeing genes involved in action. However, tsetse fed trypanosomes would be a mixture of self-cured and infected flies at day 3 and since we are unable to decipher the infection status of these flies based on the 454 data, we refer to these flies as 'flies fed or challenged with trypanosomes.'

Serine proteases and inhibitors: Serine proteases possess clip domains (at least one disulfide-bridged structure) which can potentially activate immune response pathways including the prophenoloxidase pathway and lead to the melanisation of pathogens and parasites (Jiang *et al.* 1998; Jiang *et al.* 1999; Kanost 1999; Jiang and Kanost 2000; Jiang *et al.* 2011). A serine protease was identified to be up regulated in tsetse fed trypanosomes. Phenoloxidase activity has been reported in the haemolymph of tsetse flies infected with trypanosomes (Nigam *et al.* 1997). In order to prevent increased levels of activation of protease cascades involved in immunity pathways, serpins (serine protease inhibitors) play essential regulatory roles (Jiang *et al.* 1999; Kanost 1999; Jiang and Kanost 2000). We identified the down regulation of protease inhibitors: two serine proteinase inhibitors, a metalloendopeptidase (part of the protease family) and one chymotrypsin inhibitor in flies fed trypanosomes. In blood-sucking insects, serine proteinase inhibitors block the activation of the host's blood-clotting cascades (Gubb *et al.* 2010). In *Drosophila,* serpins regulate the Toll-mediated defence pathway (serpin43Ac) (Levashina *et al.* 1999) and the melanin proteolytic cascade (serpin27A) (De Gregorio *et al.* 2002). In invertebrates, serine proteases and serpins have been implicated in antifungal immune response system and the melanisation pathway (Cerenius and Soderhall 2008). Proteinases are secreted by pathogenic parasites which help them to escape host immune defences by either inactivating or causing unsuitable expression of host immune responses (Armstrong 2001). Thus host proteinase inhibitors can inactivate the potent proteinase factors of pathogens,

protecting the host from pathogenic infections (Kanost 1999; Armstrong 2001). *T. b. brucei* contain several proteinases which can be released into the bloodstream of their infected hosts (Troeberg *et al.* 1996). In *Drosophila,* serpins (serpin42Da) inhibit chymotrypsin, a proteolytic enzyme, generally induced upon blood feeding and part of the digestive cascade started by trypsin activation (Vizioli *et al.* 2001; Yan *et al.* 2001). In tsetse gut, trypsin has been suggested to trigger bloodstream form (BSF) trypanosomes to midgut procyclics (PCFs) (Imbuga *et al.* 1992). It has been suggested that early trypsins and chymotrypsins may trigger transcription of serine proteases (Barillas-Mury *et al.* 1995; Noriega *et al.* 1995; Vizioli *et al.* 2001). Similarly, *P. gallinaceum* uses trypsin from the gut of *Ae. aegypti* to secrete chitinase, which helps the parasites traverse the peritrophic matrix (Barillas-Mury *et al.* 1995; Noriega *et al.* 1995; Shahabuddin *et al.* 1996).

Adhesion proteins: We identified the down regulation of a chitinase gene and mucin related glycoproteins in tsetse challenged with trypanosomes. The chitinase enzyme degrades chitin, a component of the peritrophic matrix (PM) of tsetse (Yan *et al.* 2002). The tsetse PM is composed of a mixture of glycosaminoglycans, glycoproteins and chitin (Lehane *et al.* 1996). Chitinase genes have been characterized in parasites including *Plasmodium* (Vinetz *et al.* 2000) and *Leishmania* (Shakarian and Dwyer 1998). Mucin is also a constituent of tsetse PM and a peritrophin, thus also potentially playing a role in the midgut (Theopold *et al.* 1996; Lehane 1997). The down regulation of genes comprising the PM suggests that tsetse flies infected with trypanosomes prevent these genes from degrading the PM and prevent trypanosomes from traversing the PM. Gibson and Bailey (2003) observed trypanosomes confined within the PM in the midgut between 1 and 3 days post infectious bloodmeal.

Reactive intermediates: We identified the down regulation of various genes involved in oxidation-reduction in tsetse fed trypanosomes: alcohol dehydrogenase, carbon-nitrogen hydrolase, and homogentisate-dioxygenase. Reactive oxygen species (ROS) play a role in insect immunity (Kumar *et al.* 2003; Molina-Cruz *et al.* 2008; Kumar *et al.* 2010) and antioxidant genes respond to midgut trypanosome infections (Munks *et al.* 2005). In this analysis, the down regulation of these antioxidant genes which are involved in reduction of free radicals suggest that the free radicals generated from ROS in tsetse challenged trypanosomes can freely form further reactive molecules with anti-parasitic properties to combat the infection.

Although we have shown an up regulation of NOS in flies exposed to trypanosomes in chapter 2, NOS was not up regulated in the current data set. However, in chapter 2 midguts were dissected at day 6 post-infection (infected at first bloodmeal) compared to the transcriptome data where midguts were dissected day 3 post-infection (infected at fourth bloodmeal). Hence, the timing of infection and dissection is important in induction of antioxidant genes. In addition, the 454 data represents a mixture of self-cured and infected flies while in chapter 2, NOS was up regulated in infected flies.

Iron metabolism and ion transporters: In hematophagous insects, the host bloodmeal provides iron which is an essential nutrient and a powerful toxin (Strickler-Dinglasan *et al.* 2006). Iron is required for oxidative metabolism, however an excess of iron can catalyze the formation of free radicals, leading to oxidative stress, causing cellular damage to the host and possibly influencing the fitness of symbionts and parasite survival in the fly (Attardo *et al.* 2006; Strickler-Dinglasan *et al.* 2006). Transferrin, a member of iron-binding proteins and part of a family of 80 kDa glycoproteins, helps sequester iron, thereby decreasing the impending oxidative stress (Attardo *et al.* 2006; Geiser and Winzerling 2011). Transferrin plays various physiological roles in development, reproduction, homeostasis and immunity (Strickler-Dinglasan *et al.* 2006; Guz *et al.* 2007). Following bacterial immune challenge, transferrin transcripts are up regulated in *Ae. aegypti* (Yoshiga *et al.* 1997)*, Bombyx mori* (Yun *et al.* 1999) and *Drosophila* (Yoshiga *et al.* 1999). Transferrin is up regulated in gut and carcass of *An. gambiae* infected with *P. falciparum* and *P. berghei* (Dong *et al.* 2006), in *Cx. quinquefasciatus* infected with *Wuchereria bancrofti* filarial worms (Magalhaes *et al.* 2008b). However *Ae. aegypti* infected with Chikungunya virus results in a down regulation of transferrin protein (Tchankouo-Nguetcheu *et al.* 2010). Tsetse flies with stable midgut trypanosome infection *(T. b. brucei)* express lower transcript levels of transferrin than uninfected naive flies, while self-cured flies have close to normal transferrin transcript levels (Guz *et al.* 2007; Lehane *et al.* 2008). We identified the down regulation of transferrin in flies challenged with trypanosomes consistent with observations by Guz *et al* (2007) and Lehane *et al* (2008). This suggests that trypanosomes in infected flies might be influencing transferrin gene expression in the host to offer a nutritionally enhanced midgut environment, which includes more iron which subsequently results in higher levels of free radicals (Guz *et al.* 2007; Lehane *et al.* 2008).

Two transmembrane transporters were identified in the list of down regulated genes (SLC26 sulfate transporter and monocarboxylate SLC16 transporter). However, apart from transport processes, the function of these genes in relation to immunity is unknown.

Immune-related genes: We identified the down regulation of a secretory phospholipase, potentially phospholipase A2 (PLA_2), which is stimulated by bacterial challenge and acts as part of insect immune defences (Stanley 2006). Inhibition of secretory PLA_2 has been suggested to suppress insect immune reactions (Stanley 2006). Infections with *T. rangeli* leads to the inhibition of PLA_2 in *Rhodnius prolixus,* although whether the parasites directly inhibit PLA_2 in their hosts could not be determined (Garcia *et al.* 2004). The expression of aquaporin (aquaporin-2), involved in ion transport processes, is up regulated after *Ae. aegypti* are infected with alphaviruses such as Sindbis virus (Sanders *et al.* 2005). In female *Ae. aegypti,* in addition to blood feeding, gene expression of lipophorin (a lipid carrier protein) is activated upon infections with Gram-positive bacteria, fungal spores and *P. gallinaceum* (Cheon *et al.* 2006). We observed the down regulation of both aquaporin and lipophorin in tsetse challenged with trypanosomes, possibly due to a high number of flies eliminating the trypanosomes, leading to a decrease in these genes known to be induced upon immune challenge. Alpha glucosidase, involved in carbohydrate metabolism has been suggested to favour virus survival and replication in midguts of *Ae. aegypti* (Tchankouo-Nguetcheu *et al.* 2010). We identified one putative alpha glucosidase gene, suggesting that perhaps infected tsetse flies down regulate the gene's expression to inhibit parasite survival in the midgut.

A putative acid sphingomyelinase gene was up regulated in flies challenged with trypanosomes. Acid sphingomylinase plays a role in apoptosis and stress signalling (Won and Singh 2006). Sphingolipids are generated by sphingomyelinases and the secondary messengers of sphingolipids, ceramide mediate cellular redox homeostasis (through the regulation of NADPH oxidase and NOS (Won and Singh 2006)) and act as effector molecules in apoptosis (Kolesnick 1998). It has been suggested that pathogenic bacteria secrete sphingomyelinases which in turn induce endogenous ceramide by host cells (Abboushi *et al.* 2004). Thus it is possible that increased expression of acid sphingomyelinase leads to increased ceramide, causing apoptosis of trypanosomes in tsetse.

We identified a putative farnesoic acid O-methyltransferase gene which is part of the juvenile hormone biosynthetic pathway, catalyzing methylation of farnesoic acid to methyl

farnesoate (Burtenshaw *et al.* 2008). Juvenile hormone regulates immunity in *Drosophila* by decreasing the expression of the antimicrobial peptide, diptericin (Burtenshaw *et al.* 2008). We also identified the up regulation of a gene involved in olfaction (smell impaired 21F). This gene is up regulated in *Varroa*-tolerant bees compared to sensitive bees (Navajas *et al.* 2008).

Stress response genes: We identified six heat-shock proteins (HSPs) which were up regulated in flies challenged with trypanosomes. Expression of HSP70 has been shown to be up regulated in female *An. gambiae* infected with o'nyong-nyong virus and has been suggested to interrupt viral replication (Amici *et al.* 1994). Heat shock proteins are also induced by oxidative stress (Tower 2011). Thus it is likely that the up regulation in HSPs identified in our data set is due to oxidative stress caused by the parasitic challenge.

In summary, the 454 data suggests that a number of genes are differentially regulated during the process of a trypanosome infection in tsetse flies. The pattern rising from this analysis suggests that trypanosome infection has a substantial effect on metabolic and cellular processes in the tsetse fly. The common classes emerging from the analysis include serine proteases and serpins, adhesion proteins involved in tsetse PM composition, reactive intermediates, immune-related genes and genes of unknown function. The identification of differentially expressed genes, particularly ones not known to be involved in immunity suggests the limitations of data generated from 454 sequencing which is based on a limited number of contigs. Quantitative PCR (qPCR) reaction should be carried out to validate the differentially expressed genes. In addition, transcriptome analysis of more replicates are needed to accurately identify differentially expressed genes.

5.4.3 Extension of gene sequences

We also attempted to extend the sequences of 16 antioxidant genes (Table 5.2) using the 454 cDNA sequence. However, we were unable to extend the sequences further as the length of the protein sequence was of similar length to the contig's protein sequence, therefore no further extension was necessary.

An early edition of the *G. m. morsitans* genome sequence became available (gmorsitans.CONTIGS-Yale.GmorY1.fa) and this was used to extend nucleotide and protein sequences of NOS, Duox and OXR1. The original NOS sequence retrieved from GeneDB consisted of 234 amino acid region encompassing the reductase domain. We have now extended the NOS sequence to incorporate conserved domains and specific catalytic units,

reductase and oxygenase, which together provide the comprehensive mechanism required for the production of nitric oxide. The C-terminal reductase domain can bind to NADPH, FAD and FMN, while the N-terminal oxygenase domain binds to haem and BH_4 (used for dimerisation), and can convert the reaction intermediate N^G-hydroxy-L-arginine to nitric oxide and L-citrulline (Figure 5.6) (Andrew and Mayer 1999). A calmodulin (CaM) binding domains connects these two regions and plays a role in the structural and functional characteristics of NOS (Andrew and Mayer 1999; Crane *et al.* 2010).

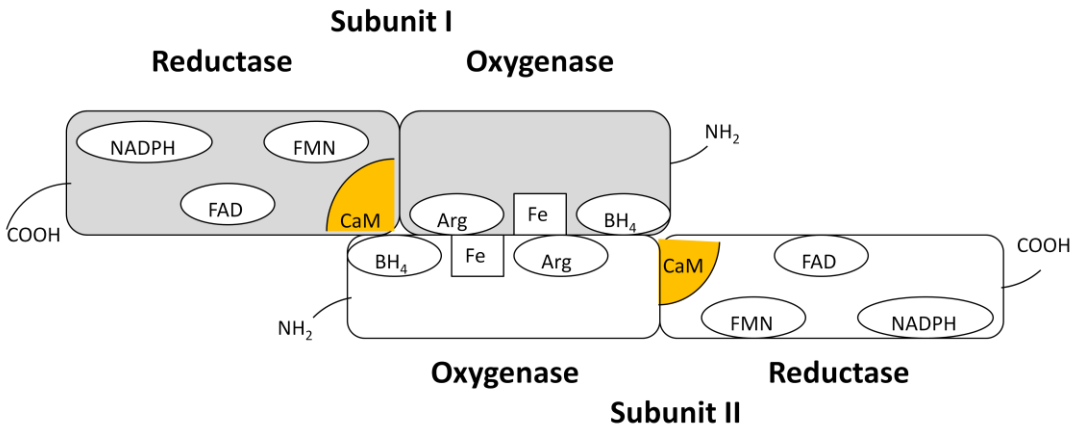


Figure 5.6. Schematic representation of the domain structure of the NOS dimer. NOS consists of two similar monomers, divided into a C-terminal reductase domain and an N-terminal oxygenase domain. A calmodulin (CaM in yellow) binding domain connects these two regions. The reductase domain can bind to NADPH, FAD and FMN and the oxygenase domain can bind to haem, BH_4 and L-arginine. (Modified from: (Andrew and Mayer 1999)).

The Duox sequence was also extended from 223 amino acids to 1,215 amino acids in length, comparable to *Drosophila* Duox at 1,475 amino acids in length. The newly extended sequence now encompasses the large N-terminal peroxidase-homology domain, the dual peroxidase domain (residues 1-538). Peroxidases are suggested as enzymes which can catalyze the formation of di- and trityrosine bonds in the presence of hydrogen peroxide, and the dityrosine network has recently been shown to protect microbiota by preventing the activation of epithelial immunity in midguts of *An. gambiae* (Donko *et al.* 2005; Kumar *et al.* 2010). The human genome contains a few NADPH oxidase famly members (Nox 1-5 and Duox 1-2) (Ha *et al.* 2005a). In addition to the phagocytic oxidase complex ($gp91^{phox}$), the N-terminal extracellular peroxidase-homology domain helps distinguish the Duox family from the Nox family (Ha *et al.* 2005a). Ha *et al.* (2005) have shown that the peroxidase domain displays antimicrobial effects in the presence of chloride and hydrogen peroxide, suggesting that this domain induces bacterial death depending on chloride concentration

and is required for the microbicidal effects of Duox. The extended protein also contains a pair of calcium binding motifs (residues 772-834) which is important for inducing conformational change in the EF-hand motif, and binds to the catalytic domain inducing electron transfer from NADPH to molecular oxygen to form superoxide anion (Rubbo *et al.* 1994; Donko *et al.* 2005). Superoxide anion can react with other reactive intermediates to form potentially toxic radicals.

Finally, we have successfully managed to extend the OXR1 protein and nucleotide sequences. The protein sequence has been extended to 730 amino acids, comparable to *Drosophila's* OXR1 longer isoform of 789 amino acids. The *D. melanogaster* OXR1 gene (CG32464) has undergone alternative splicing of 25 exons to generate 19 different transcripts (Jaramillo-Gutierrez *et al.* 2010). However, the most conserved region is the carboxyl-terminal TLDc domain restricted to eukaryotes and usually associated with lysin motifs (LysM) as shown in the OXR1 extended sequence of tsetse.

5.4.4 Summary

This chapter has shown the phylogenetic relationships of tsetse NOS, Duox and OXR1 to other dipterans and vertebrates. In addition, the 454 midgut transcriptome data suggests that a number of genes are differentially regulated when tsetse flies are exposed to trypanosome infection. Finally, we have used the latest available tsetse genome sequence to manually extend nucleotide and protein sequences of NOS, Duox and OXR1 to include major domains that are crucial for the genes' functionality.

CHAPTER 6

Manual tsetse genome annotation of antioxidant genes

6.1 Introduction

Tsetse flies are obligate blood feeders ingesting large volumes of vertebrate blood which are several times their unfed body weight (Munks *et al.* 2005). The biochemical nature of vertebrate blood creates a physiological challenge for the fly. One mode of detoxification involves the expression of antioxidant enzymes (Paes *et al.* 2001; Jaramillo-Gutierrez *et al.* 2010) such as superoxide dismutase (SOD) and catalase which convert highly reactive species into lesser or non-reactive species. In addition, immune defences against trypanosomes in tsetse are believed to include the generation of superoxides, thereby increasing the impending oxidative stress in the tsetse midgut (Hao *et al.* 2003). Consequently, antioxidant genes probably play a significant regulatory role in midgut physiology of tsetse flies due to defences against bloodmeal digestion and trypanosomes (Munks *et al.* 2005). The recent release of the *Glossina morsitans morsitans* genome provides an opportunity to manually annotate some of the previously identified tsetse antioxidant genes (Lehane *et al.* 2003; Munks *et al.* 2005; Attardo *et al.* 2006) along with some newly identified antioxidant genes found in other insects (Ohno *et al.* 1994; Gerardo *et al.* 2010; Jaramillo-Gutierrez *et al.* 2010).

The Wellcome Trust Sanger Institute, part of the International *Glossina* Genomics Initiative (IGGI), has taken a whole genome shotgun sequencing (WGS) approach to the tsetse fly, *G. m. morsitans* (Yale colony, USA). The development of Sanger Sequencing, also known as the dideoxynucleotide (ddNTP) or chain termination method by Frederic Sanger in 1975 has allowed genome sequencing of various organisms including the human genome (Venter *et al.* 2001) and the malaria vector, *Anopheles gambiae* (Holt *et al.* 2002). This method fragments DNA template into smaller molecules which are then cloned into a plasmid vector used to transform *E. coli* (Shendure and Ji 2008). In each sequencing cycle a single bacterial colony is used to isolate plasmid DNA and each sequencing cycle reaction generates a ladder of ddNTP-terminated, dye-labeled products which are separated by high-resolution electrophoresis within capillaries (Shendure and Ji 2008). A four-channel emission spectrum generates a sequencing trace as the fluorescently labelled fragments of distinct sizes pass through a detector (Shendure and Ji 2008). Software translates the traces into a DNA sequence. Alternatives for faster and cheaper DNA sequencing are now present, although the mechanisms behind them are still fundamentally based on the Sanger sequencing.

In 1995, the genome sequence of a living species, *Haemophilus influenza* via WGS was released (Fleischmann *et al.* 1995). This sequence was only 1.8 million base pairs in length and required 4 months of sequencing to produce (Fleischmann *et al.* 1995; Venter 2011). Changes in automation and parallel DNA sequencing resulted in the first release of the human genome sequence in 2001 which was 3 billion base pairs in length and took only 9 months to produce, suggesting a > 1000-fold improvement (Venter *et al.* 2001). Currently, single DNA instruments have the capacity to produce 100 million base pairs per day demonstrating the implications of improved changes to automation and massively parallel DNA sequencing with increased productivity rates by a large order of magnitude (Venter 2011).

Genomic information from the *Glossina* sequencing data provides researchers with genetic information important for vector competence, haematophagy, single-nucleotide polymorphisms (SNPs) and genotyping for population level studies and genome wide association studies relevant to *Glossina*-trypanosome biology (Alves-Silva *et al.* 2010). After the assembly process, computational or automatic annotation was performed at VectorBase, however biologists with their area of expertise were required to confirm and check the results for reliability. The results from computational analyses were made accessible via visualization tools to allow annotators to identify transcripts based on EST and protein sequence similarities, RNAseq data (Illumina), and gene predictions from Augustus (SOLiD), SNAP and GenScan.

Most annotated genes are at least partly covered by predictions. However, sometimes gene prediction tools are unable to detect nested genes (genes located inside the introns of other genes) (Salamov and Solovyev 2000). In addition, gene prediction tools are much worse at predicting terminal exons than internal ones, resulting in some actual genes being split up and/or other multiple genes being joined into a single gene (Salamov and Solovyev 2000). Therefore using the results of the automated annotation as a baseline, manual curation of genes of biological interest is needed to allow a more in-depth annotation of predicted genes and dismiss false positives (Yandell *et al.* 2005). Annotating individual predicted exons allows us to manually refine the data and identify errors which might occur with the automated annotation process.

Therefore, the aim of this chapter was to manually annotate novel and previously identified antioxidant genes in tsetse. This involved predicting transcript and protein sequences, examining exon boundaries, and adding the function of each predicted protein. After the

manual annotation, GFF (gene finding format or general feature format) files with features such as starts, stops, splice sites, exons and introns of particular genes were submitted to VectorBase. A comparative analysis with the dipteran *Drosophila melanogaster* was then carried out.

6.2 Materials and methods

6.2.1 Computational analyses

Automated annotation was compiled by Ensembl and MAKER pipelines to detect genes in the assembled *G. m. morsitans* sequence by VectorBase. MAKER aligns ESTs and proteins to a genome to produce *ab initio* gene predictions and automatic gene annotations (Ignarro *et al.* 1981). The final gene set of 12,220 genes was compiled using the MAKER pipeline using raw data to make a consensus gene set. The raw data included EST based genes and protein based genes, gene sets obtained from second-generation DNA sequencing technologies (RNAseq - Illumina and Augustus - SOLiD) and *ab initio* algorithms (SNAP, Augustus and GenScan) in which genomic DNA sequence is searched for signals of protein-coding genes. Using the results of the computational annotation as a baseline, manual annotation of antioxidant genes was subsequently carried out to refine the automated gene predictions.

6.2.2 Manual annotation

Firstly, antioxidant ortholog nucleotide gene sequences were identified by searching through the *Drosophila* database, FlyBase (http://flybase.org) or the *Anopheles* database on Vectorbase (http://agambiae.vectorbase.org/). The ortholog sequences and known EST sequences of tsetse genes from GeneDB were then downloaded to a FASTA file and then blasted against the *Glossina* supercontigs (http://gmorsitans.vectorbase.org) and positive hits were identified (e-value $1e^{-10}$). The corresponding supercontigs sequence data were then downloaded to a FASTA file while the GFF data for the supercontig was downloaded from a data portal (http://www.vectorbase-cap.ensemblgenomes.org/). The Apollo browser (http://apollo.berkeleybop.org/) was used to load the supercontig and the associated GFF file. The automated feature predictions were compared with BLAST results and features corresponding to the ortholog were identified. Predicted gene features were analyzed and the final changes were saved to a GFF file and submitted to VectorBase via the data portal.

6.1.3 Exon-intron boundaries

The Apollo browser was used to visually analyze exon-intron boundaries using raw data, gene models and data from *ab initio.* Exon-intron boundaries and splice variants were then compared to the *Drosophila* ortholog (www.flybase.org).

6.3 Results

6.3.1 Characteristics of the tsetse genome

The genome size of *Glossina* was compared to *Aedes, Anopheles, Culex* and *Drosophila.* Figure 6.1 depicts the genome size (Mb) with the total tsetse genome size being 365 Mb compared to *Drosophila's* genome size of 168 Mb. Table 6.1 lists the characteristics of the tsetse genome compared to other insects. The number of exons in tsetse and the number of predicted genes are similar to the other species, while the average size of *Glossina's* genes were longer compared to *Anopheles, Culex* and *Drosophila.*

6.3.2 Antioxidant gene list

A list of antioxidant genes was compiled for manual annotation. Table 6.2 lists the genes, their symbols, their *Drosophila* homologs, their putative functions and their location on the *Glossina* genome. The genes include nitric oxide synthase (NOS), dual oxidase (Duox) and oxidation resistance 1 (OXR1) (chapters 2 and 4), along with immune-regulated catalase, catalase, superoxide dismutases, thioredoxin reductases, thioredoxin peroxidases and prophenoloxidase.

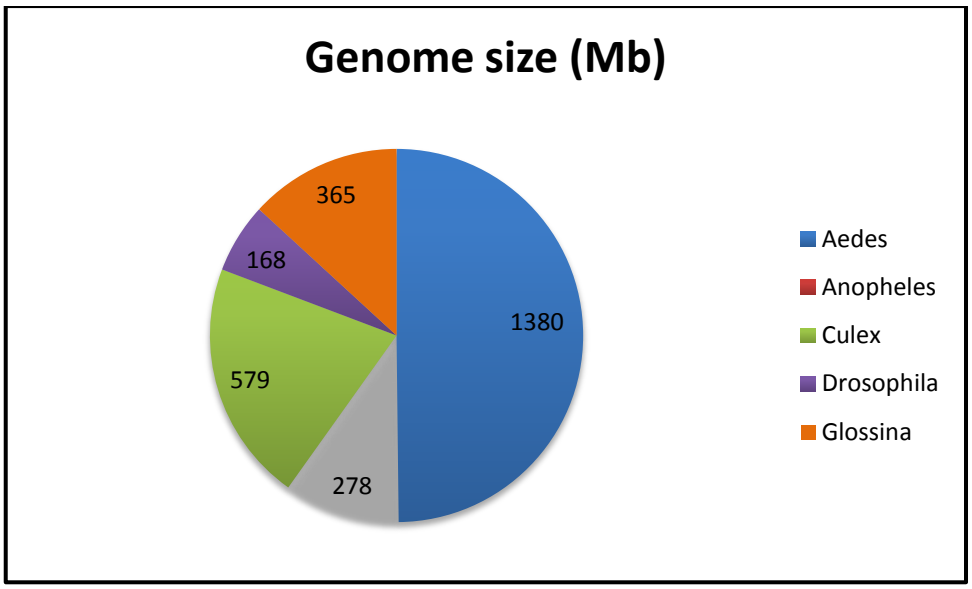


Figure 6.1. Genome size (Mb) of *G. m. morsitans* compared to *Aedes, Anopheles, Culex* and *Drosophila*.

Table 6.1. Characteristics of the *G. m. morsitans* genome compared to *Aedes, Anopheles, Culex* and *Drosophila*.

Genome features	*Aedes*	*Anopheles*	*Culex*	*Drosophila*	*Glossina*
Number of predicted genes	15,419	12,457	18,883	14,039	12,220
Number of exons	63,650	52,595	71,094	65,706	64,464
Number of introns	51,076	38,051	52,211	43,588	52,466
Average gene size	15,488 bp	5,145 bp	5,673 bp	5,253 bp	9,386 bp

Table 6.2. Summary of tsetse *(G. m. morsitans)* antioxidant gene annotation. Gene localization based on supercontig assembly.

Gene name	Symbol	*Drosophila* ortholog	Putative functions	Amino acids	Location	Introns
Nitric oxide synthase	Nos	CG6713	Produces NO	1,307	Scf7180000645283	16
Dual oxidase	Duox	CG3131	Peroxidase activity (generates H_2O_2); defense response to bacterium; mucosal immune response	1,215	Scf7180000652170	5
Oxidation resistance 1	OXR1	CG32464	Regulates basal levels of catalase	1,271	Scf7180000651117 Scf7180000651426	19
Immune-regulated catalase	IRC	CG8913	Oxidation-reduction process; response to oxidative stress	Not found		
Catalase	Cat	CG6871	Removes H_2O_2; detoxifying enzyme	487	Scf7180000647982	0
Superoxide dismutase 1	Sod1	CG11793	Response to oxidative stress	Partial	Scf7180000651410	Not determined
Superoxide dismutase 2	Sod2	CG8905		189	Scf7180000644939	1
Superoxide dismutase 3	Sod3	CG9027		Not found		
Thioredoxin reductase-1	Trxr-1	CG2151	Cell-redox homeostasis; H_2O_2 catabolic process	490	Scf7180000646622	0
Thioredoxin reductase-2	Trxr-2	CG11401		249	Scf7180000648298	0
Thioredoxin peroxidase-1	Jafrac1	CG1633		194	Scf7180000650867 Scf7180000648834	1
Thioredoxin peroxidase-2	Jafrac2	CG1274		231	Scf7180000639967	2
Thioredoxin peroxidase-2	Jafrac-3	CG5826		220	Scf7180000652017	3
Prophenoloxidase	CG42640	CG42640/CG2952	Phagocytosis & encapsulation	1,016	Scf7180000644323	4

6.3.3 Exon-intron boundaries

Raw data, gene models and data from *ab initio* algorithms were used to visually analyze exon-intron boundaries using the Apollo browser. This was then compared to the *Drosophila* ortholog.

Nitric oxide synthase (NOS): The *Glossina* NOS gene consists of 16 splice sites to generate one transcript made up of 17 exons. The number of exons is comparable to the longest NOS transcript (NOS-RB) of *D. melanogaster* NOS (10 annotated transcripts and polypeptides) which consists of 25 exons including 5' and 3' untranslated regions while *An. stephensi* NOS consists of 19 exons. Tsetse has one NOS homolog consistent with other insects. It is 1,307 amino acids in length with 73% identity to the *D. melanogaster* NOS and 63% identity to *An. stephensi* NOS. The full length *Glossina* NOS also includes all important domains necessary for a fully functional NOS protein, including the oxygenase and reductase domains. Figure 6.2 (Panels A and B) shows the fully annotated *Glossina* NOS and the *D. melanogaster* ortholog, along with the conserved domains. The predicted mRNA structure is similar to *D. melanogaster* while the conserved amino acids of the oxygenase domains spans exons 1-9 in tsetse and exons 1-11 in *D. melanogaster*. The FMN binding domain spans exons 10-14 in tsetse and exons 12-16 in *D. melanogaster* and the FAD binding domain spans exon 15 in tsetse and exons 17-19 in *D. melanogaster.* The reductase domain spans exons 15-16 in tsetse and exons 17-19 in *D. melanogaster.*

Dual oxidase (Duox): Duox (Figure 6.2 Panels C and D) was manually annotated onto supercontig scf7180000652170 of the *Glossina* genome. The *Glossina* Duox gene consists of six exons compared to 10 exons of the only Duox transcript found in *D. melanogaster* (CG3131) in addition to 5' and 3' untranslated regions. The *Glossina* Duox gene consists of 1,215 amino acids in length compared to Duox of *D. melanogaster* of 1,537 amino acids in length with 91% identity to each other in protein sequence. The *Glossina* Duox contains the dual peroxidase domain (exons 1-3), the ferric reductase domain (exon 3), calcium-binding domains (exons 4-5) and FAD-binding domain (exon 6).

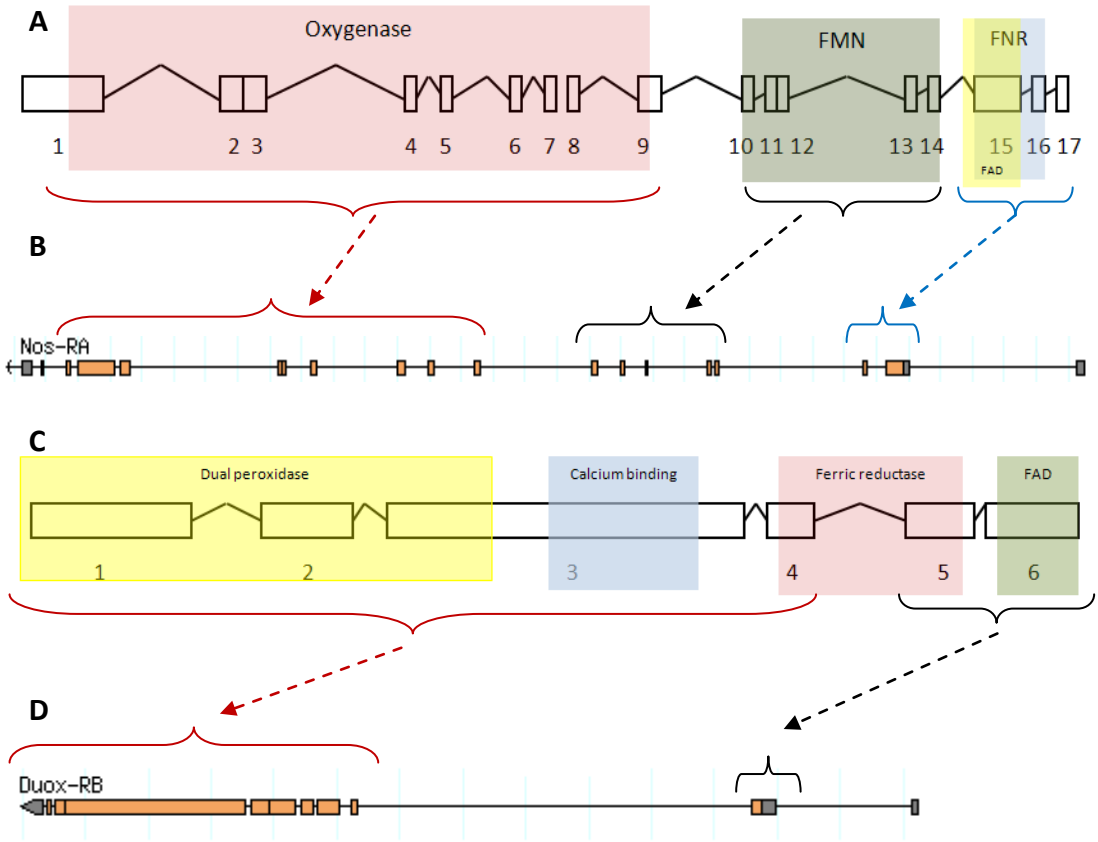


Figure 6.2. Exon-intron boundaries of *Glossina* NOS (A) and *D. melanogaster* NOS (B) and *Glossina* Duox (C) and *D. melanogaster* Duox (D). The longest NOS transcript of *Drosophila* was chosen (CG6713; Nos-RA) to compare the two NOS orthologs while only one Duox transcript is present in *D. melanogaster* (CG3131). The regions of each conserved domains are highlighted.

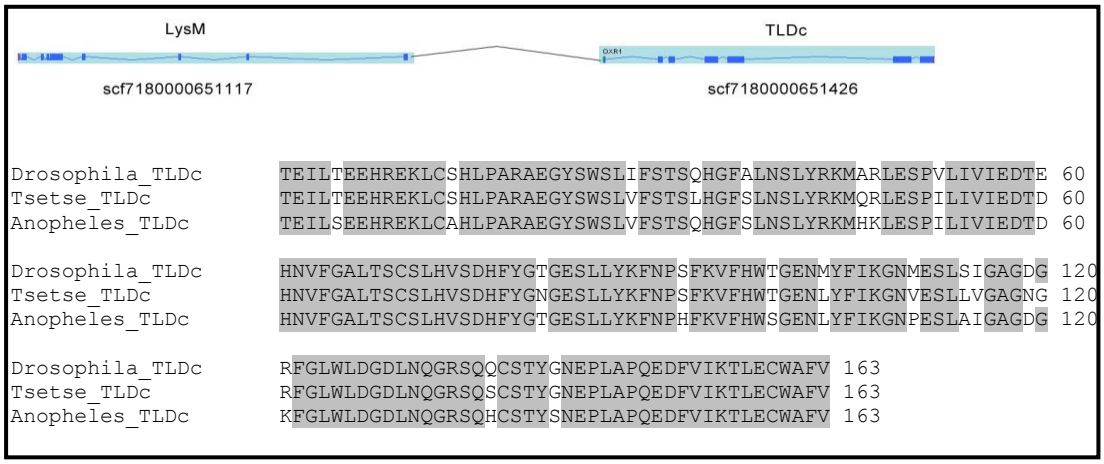


Figure 6.3. Exon-intron boundaries of *Glossina* OXR1 gene and its conserved domains and alignment of the highly conserved TLDc domains of *G. m. morsitans, D. melanogaster* (CG32464) and *An. gambiae* (AGAP001751-PA).

Oxidation resistance 1 (OXR1): The OXR1 gene (CG32464) in *D. melanogaster* has 19 different transcripts as a result of alternative splicing of 25 different exons (FlyBase and Entrez Gene). The OXR1 gene (AGAP001751-PA) in *An. gambiae* consists of at least 17 exons and contains at least three different mRNA transcripts (Jaramillo-Gutierrez *et al.* 2010). Through manual annotation, the *Glossina* OXR1 gene (1,271 amino acids in length) was identified to span two supercontigs (scf7180000651117 and scf7180000651426) covering the lysin and TLDc domains respectively. The full length assembled transcript spans 20 exons, with 13 exons in the first supercontig (scf7180000651117) and 7 exons in the second supercontig (scf7180000651426) as a result of splicing events. Figure 6.3 shows the exon-intron boundaries of OXR1 of *G. m. morsitans* and the carboxyl-terminal TLDc region alignment of *G. m. morsitans, An. gambiae* and *D. melanogaster*. The full length protein sequence of *Glossina* OXR1 has 67% homology to the protein sequence of *D. melanogaster* OXR1 and 52% homology to the *An. gambiae* OXR1 while the TLDc region was the most highly conserved region of the OXR1 gene in all three species. The TLDc region of *G. m. morsitans* was 92% and 90% identical to TLDc regions of *D. melanogaster* and *An. gambiae* respectively in amino acid sequences.

Immune-regulated catalase (IRC): The *D. melanogaster* ortholog (CG8913) was used to search the tsetse genome, however the resulting hits did not align with the *D. melanogaster* IRC. No *Glossina* IRC gene was found.

Catalase: The deduced amino acid sequence of *Glossina* catalase is 81% identical to catalase of *D. melanogaster* (CG6871) and has 70% identity to the other *D. melanogaster* homolog CG9314. Only one transcript of tsetse catalase was found in the genome, with no splicing events compared to *Drosophila's* three exons in addition to untranslated regions.

Superoxide dismutase 1 (SOD1): Five SODs are present in *D. melanogaster* and these were used to search the tsetse genome. The *D. melanogaster* ortholog of SOD1 (CG11793) was found in the *Glossina* genome although it is only a partial sequence. The *Glossina* ortholog of SOD1 was aligned to the two transcripts of *D. melanogaster* SOD1 and *An. gambiae* SOD1. The deduced amino acid was 85% and 88% identical to the two transcripts of SOD1 from *D. melanogaster* and 89% and 92% identical to SOD1 from *An. gambiae,* AGAP007497 and AGAP010517 respectively.

Superoxide dismutase 2 (SOD2): The superoxide dismutase 2 (SOD2) from *D. melanogaster* (CG8905) was searched in the tsetse genome and the deduced tsetse amino acid sequence was 67% identical to the only transcript of SOD2 from *D. melanogaster.* The *Glossina* SOD2 spans 2 exons.

Superoxide dismutase 3 (SOD3): The *D. melanogaster* ortholog (CG9027) was used to search the *Glossina* genome, however the resulting hits did not align well. No SOD3 was found in tsetse.

Thioredoxin reductase-1 (Trxr-1): *D. melanogaster* contains three transcripts of Trxr-1. The *Glossina* Trxr-1 was identified on the tsetse genome, with only a single homolog found spanning one exon only. The deduced amino acid sequence is 51% identical to the amino acid sequences of the three *D. melanogaster* transcripts which consist of 8 exons of which 4 are untranslated regions.

Thioredoxin reductase-2 (Trxr-2): The *D. melanogaster* Trxr-2 (CG11401) has one transcript spanning two exons of which one is untranslated. The deduced *Glossina* amino acid sequence (spanning one exon only) has 68% identity to the *D. melanogaster* gene.

Thioredoxin peroxidase-1 (Jafrac-1): The *D. melanogaster* Jafrac-1 gene (CG1633) has 2 annotated transcripts which were used to search for a *Glossina* ortholog. Two transcripts of the Jafrac-1 gene were found in the *Glossina* genome, on supercontig scf7180000650867 and scf7180000648834. The amino acid sequences from the two supercontigs were 79% identical to the protein sequences of the two transcripts of *D. melanogaster* ortholog.

Thioredoxin peroxidase-2 (Jafrac-2): The *D. melanogaster* Jafrac-2 gene (CG1274) has 2 transcripts and both transcripts are 76% identical to the *Glossina* ortholog with contains 3 exons compared to 7 exons including 3 untranslated regions of *D. melanogaster.*

Thioredoxin peroxidase-3 (Jafrac-3): A third thioredoxin peroxidase (Jafrac-3) was found in the *Glossina* genome on supercontig scf7180000652017 with 220 residues in length and spanning 4 exons, with 62% identity in amino acid sequence to a peroxiredoxin 3 of *D. melanogaster* (gi: 17738015).

Prophenoloxidase (PPO): The *D. melanogaster* PPO ortholog (CG42640) was used to initially search for a PPO gene in tsetse. Although a supercontig hit was retrieved, the amino acid similarity between *Drosophila* and tsetse was very poor (~3%).

6.4 Discussion

Reactive oxygen species (ROS) are frequently generated as by-products of redox reactions. However, excess generation of ROS leads to oxidative damage and thus, enzymatic and non-enzymatic antioxidant systems are in place to avoid the toxic effects of an oxidative challenge. The obligate blood feeding nature of tsetse flies creates a physiological challenge to the flies. The pro-oxidant activity of haem released from blood can act as a catalyst to generate ROS (Oliveira *et al.* 2011). ROS can damage DNA, proteins and lipids, inactivate enzymes and disrupt cellular membranes (Schmidt *et al.* 1993; Oliveira *et al.* 2007). One mode of coping with pro-oxidant effects of ingested haem in arthropods is the sequestration of haem into haemozoin, a less reactive molecule (Oliveira *et al.* 2000b). Another mode of detoxification in arthropods including tsetse involves the expression of antioxidant enzymes (Paes *et al.* 2001; Jaramillo-Gutierrez *et al.* 2010) such as superoxide dismutase (SOD) which converts superoxide anion into hydrogen peroxide and catalase which converts hydrogen peroxide into water and oxygen. In addition, in response to trypanosomes, tsetse flies generate superoxides, which induce oxidative stress in the tsetse midgut (Hao *et al.* 2003). As a result, antioxidant genes most likely play an important regulatory role in the physiology of tsetse midguts due to defences against trypanosomes and bloodmeal digestion (Munks *et al.* 2005). The recent release of the *G. m. morsitans* genome (365 Mb) provides an excellent opportunity to identify the components of the tsetse antioxidant system and to conduct a comparative analysis with the dipteran, *D. melanogaster.* We identified 13 antioxidant genes in the tsetse genome, these include previously identified genes in tsetse (Lehane *et al.* 2003; Munks *et al.* 2005; Attardo *et al.* 2006) and novel genes identified in other insects (Ohno *et al.* 1994; Gerardo *et al.* 2010; Jaramillo-Gutierrez *et al.* 2010).

In general, the antioxidant genes encode small proteins of approximately 295 amino acids in length, with the exception of NOS, Duox, OXR1 and prophenoloxidase (PPO) which were less than 1,307 amino acids in length. The antioxidant genes identified on the *Glossina* genome are similar in structure, including exon-intron boundaries to *Drosophila.* This is particularly true for NOS, Duox and OXR1, consistent with phylogenetic analysis performed on amino acid sequences of these genes in Chapter 5.

The enzyme NOS is up regulated in response to parasite (Luckhart *et al.* 1998; Rivero 2006) and Gram-negative bacterial infection and mediates the production of nitric oxide (NO) (Foley and O'Farrell 2003). Two NOS genes have been reported in *Bombyx mori* while a

single NOS gene exists in *Drosophila, Anopheles, Apis* and *Manduca* and we have now identified one NOS homolog in tsetse. The structural features of *Glossina* NOS are similar to both *D. melanogaster* NOS and *An. stephensi* NOS. The full length *Glossina* NOS isoform contains two similar monomers which can be divided into two major domains, the C-terminal reductase domain and an N-terminal oxygenase domain (Andrew and Mayer 1999). The C-terminal reductase domain can bind to NADPH, FAD and FMN, while the N-terminal oxygenase domain binds to haem and tetrahydrobiopterin (BH_4), and the L-Arginine substrate (Andrew and Mayer 1999; Daff 2010). A calmodulin (CaM) binding domain connects these two regions, and this CaM binding domain plays an important role in both the structure and function of NOS (Andrew and Mayer 1999; Crane *et al.* 2010).

One Duox gene in tsetse was identified, consistent with *Drosophila.* The enzyme Duox is responsible for the generation of ROS in response to oral ingestion of bacteria by *Drosophila* (Ha *et al.* 2005a). After ROS generation via Duox, ROS is eliminated by immune-regulated catalase (IRC), a secretory antioxidant enzyme with catalase activity which removes any remaining luminal ROS which might harm gut epithelia (Ha *et al.* 2005b). Although an IRC gene exists in *Drosophila,* an IRC gene in tsetse could not be identified, consistent with *An. gambiae* which also lacks an IRC gene. However, one catalase gene was identified in tsetse, consistent with *Drosophila* and *An. gambiae.* Catalase is responsible for degrading H_2O_2 into oxygen and water. *Drosophila* catalase gene contains three exons in addition to an untranslated region, while *Glossina* catalase consists of only one exon. This could possibly be due to the lack of depth of sequencing of *Glossina* in order to detect potentially more exons as the genome was constructed using raw data from RNA-seq and *ab initio* algorithms.

Oxidation resistance 1 (OXR1), an ancient gene present in all eukaryotes, regulates basal levels of the two enzymes that detoxify hydrogen peroxide (H_2O_2), catalase and glutathione peroxidase (Elliott and Volkert 2004; Durand *et al.* 2007; Jaramillo-Gutierrez *et al.* 2010). OXR1 also protects mosquitoes from oxidative stress (Jaramillo-Gutierrez *et al.* 2010). One OXR1 gene was identified, with a highly similar TLDc region, consistent with *Drosophila* and *An. gambiae.*

Superoxide dismutase (SOD) acts as a first line of defense against ROS produced in the mitochondria, converting superoxide to oxygen and H_2O_2 (Ohno *et al.* 1994). SODs generally exist in different forms depending on cellular localization and structure of their active sites (Ohno *et al.* 1994). Cu/ZnSOD (SOD1) is present in the cytoplasm while MnSOD

(SOD2) is present in the inner mitochondrial space and the extracellular SOD3 is secreted into the extracellular space (Ohno *et al.* 1994). Two SOD encoding genes were identified, SOD1 and SOD2 with high sequence homology to *D. melanogaster,* as expected. The extracellular SOD3 was not found in tsetse. This is consistent with results by Munks *et al.* (2005) who identified two SOD encoding genes (Cu/ZnSOD and MnSOD).

Thioredoxin peroxidases, also known as peroxiredoxins reduce H_2O_2 using electrons provided by thioredoxin reductase (Ohno *et al.* 1994). Three thioredoxin peroxidases were identified in tsetse, consistent with Munks *et al* (2005), and all three were highly homologous with the three 2-Cys thioredoxin peroxidases described in *Drosophila* (CG1633, CG1274 and CG5826) (Radyuk *et al.* 2001). In addition, two thioredoxin reductases were identified in tsetse while Munks *et al* (2005) only identified one thioredoxin reductase gene in tsetse. This discrepancy is due to our use of multiple raw data sets to find these genes while Munks *et al* (2005) only used EST data available at the time. Thioredoxin reductases reduce thioredoxin (important for maintaining redox homeostasis) and the resulting products act as powerful antioxidants (Ohno *et al.* 1994). Similar to *Drosophila, Glossina* has two thioredoxin reductase genes. However, *Glossina* thioredoxin reductase 1 gene consists of only one exon compared to *Drosophila's* 8 exons of which 4 are untranslated regions. Again, this could be due to the depth of sequencing of *Glossina* which was unable to identify further exons. Finally, prophenoloxidase (PPO) mediates melanin formation, leading to the encapsulation of pathogens and parasites (Nappi and Christensen 2005). In insects, the activation of PPO into phenoloxidase is through serine proteases (Soderhall and Cerenius 1998). *Drosophila* has one PPO gene, while *Anopheles* has nine PPO genes. Although a PPO gene in tsetse was identified, the homology to *Drosophila* and *Anopheles* was poor and needs to be investigated further by using RACE PCR strategy to correctly identify *Glossina* PPO.

This chapter represents a comparison of antioxidant genes between tsetse and *D. melanogaster.* The basic components of the antioxidant system appear to be conserved, with the exception of the absence of IRC in tsetse. Although the extracellular SOD3 was not found in tsetse, the other SODs exist in tsetse and are possibly enough to respond to oxidative stress. The lack of differential expansion of antioxidant gene families among *Glossina* and dipteran species reflects the close relation between *Glossina* and *Drosophila.* In addition, due to a larger set of raw data available, we are now able to validate antioxidant genes identified in the past and find novel genes such as a second thioredoxin

reductase gene in tsetse. Algorithms capable of predicting signals for a protein enable us to confirm whether the putative antioxidant genes predicted in the past are valid. Previous studies have shown the upregulation of SOD1 in self-cleared flies compared to infected flies, suggesting that it may be in response to increases in superoxides as a result of the tsetse immune response against trypanosomes (Hao *et al.* 2003; Lehane *et al.* 2003; Munks *et al.* 2005). In addition, increased expression of SOD1, catalase and the three thioredoxin peroxidases were observed in the posterior midgut in comparison with other gut regions of tsetse (Munks *et al.* 2005), suggesting their possible roles in coping with the oxidative stress generated by the release of haem from the bloodmeal. This demonstrates the significance of antioxidant protection to the blood feeding tsetse fly.

CHAPTER 7

Final Summary

This thesis sets out to investigate whether oxidant genes play a significant role in tsetse fly defence against trypanosomes. The primary objective was to test the hypothesis that superoxides are a core protective device. Two approaches were used in this study, a direct and an indirect one. The direct method involved the use of RNAi or chemical inhibitors to knock down antioxidant genes (NOS, Duox and OXR1) and examine their effects on parasite susceptibility. The indirect method involved exposing flies to different physiological conditions known to impact susceptibility, and measuring H_2O_2 levels in the appropriate tissues to see if there was a correlation between H_2O_2 levels and infection status.

This study provides strong evidence for the involvement of NOS in controlling trypanosome establishment in tsetse midgut (Table 7.1). The greatest effect is seen if NOS knockdown occurs before trypanosomes enter the midgut, suggesting NOS acts very early in the infection event. This early action of NOS explains the previous apparent discrepancies between the roles of NOS and NO in mosquitoes (Luckhart *et al.* 1998; Herrera-Ortiz *et al.* 2004) and those previously reported in tsetse (Hao *et al.* 2003) as the latter authors only investigated a later knockdown of NOS levels. Although the role of NOS with regards to parasite establishment (Chapter 2) is explored in considerable detail, in retrospect, it would have been useful to measure nitric oxide (NO) concentration by performing comparative measurements of the nitrite ion concentration by spectrophotometric assay based on the Greiss reagent (Hao *et al.* 2003). This would have provided a comprehensive view at the roles of both NOS and NO in parasite establishment, since NO is the active microbicidal compound generated through the NOS catalyzed reaction. In addition, although attempts were made to see if different life cycle stages of trypanosomes (PCFs and BSFs) influence NOS production, the PCFs showed inconsistent results because it was not possible to get the PCFs to grow well in culture. With time permitting, it would be interesting to infect flies with PCFs and see whether the same NOS response is observed as with the BSF-induced NOS response. Furthermore, the *in vivo* role of SNAP (a NOS donor) led to a contradicting phenotype of increased midgut infection prevalence, therefore understanding how SNAP is working in this system needs to be explored further. To do this NOS activity should be measured after flies are fed SNAP, to confirm whether SNAP is indeed increasing NO levels. No differences in salivary glands infections in flies fed either L-NAME or PBS were found compared to midgut infections. If time permitted it would have been interesting to inject L-NAME into the fly and look again at the two phenotypes

Table 7.1 Summary of physiological conditions that tsetse flies were exposed to and their effects on NOS or ROS (H_2O_2) or Duox.

Physiological condition	NOS	ROS	Duox
Age	N.S	N.S	
Fed	↑	↓	
Starved	↓	↑	
Male midgut	N.S	N.S	
Female midgut	N.S	N.S	
Male salivary glands	N.S	N.S	
Female salivary glands	N.S	N.S	
Serum fed		N.S	
Heat-inactivated serum fed		N.S	
Normal blood fed		N.S	
TsetseEP protein knocked down		N.S	
Control eGFP knocked down		N.S	
Flies emerging from pupae kept at 26°C	N.S	N.S	
Flies emerging from pupae kept at 30°C	N.S	N.S	
Gram-positive bacteria *(S. aureus)*	N.S	N.S	
Gram-negative bacteria *(E. coli)*	N.S	N.S	
Trypanosome-infected midgut compared to uninfected naive midgut	↑	↑	N.S.
Sodalis in vitro		N.S	
Sodalis in vivo	↓		

Significant increase in NOS or ROS or Duox

Significant decrease in NOS or ROS or Duox

Not significant (N.S) difference in NOS or ROS or Duox

Not determined

- Hypothesis: Increased (↑) NOS or ROS should correlate with decreased susceptibility and therefore decreased parasite prevalence.

because under these circumstances L-NAME concentration in salivary and midgut tissues might be expected to be comparable.

Due to time constraints, the suppression of NOS by *Sodalis* was not further explored either. It would be useful to examine whether NOS contributes to the phenotype in which *Sodalis* favours trypanosome establishment in tsetse midgut. Both NOS and ROS experiments gave non significant results when comparing males and females in relation to parasite susceptibility. However, the variability between the sexes might have become even more apparent and perhaps would have provided stronger evidence for the role of NOS if NOS activity was measured in salivary glands of both sexes of flies and flies with salivarian trypanosomes. Environmental stress such as puparial incubation temperature revealed no significant differences in NOS and ROS activities; this is possibly due to the small sample size because there was a trend of lower NOS activity, although non-significant, in flies emerging from heat shocked puparia. This reduced NOS activity may provide an explanation for the flies' increased susceptibility and the experiment merits repetition with larger sample sizes. If, as predicted, that gave a positive result then many unanswered questions remain. At what point in puparial development is temperature crucial for NOS activation? Can this susceptibility phenotype be rescued by keeping puparia at a high temperature and then transferring them to a lower temperature for 1-2 days before flies emerge? Similarly, if puparia were incubated at an even lower temperature (for example at 20°C), would this contribute to even higher NOS levels? Answering these questions would show how much influence temperature has in controlling the rate of puparial development, although distinguishing between immunity-based NOS and developmental NOS would be a challenge.

My results differ in the reported roles of ROS and NOS/NO against bacterial infections in mosquitoes and *Drosophila*. Although an explanation for these unexpected observations in tsetse may lie in the different feeding patterns of tsetse flies to other dipterans, a larger set of both Gram-positive and Gram-negative bacteria should be screened for both NOS and ROS activities. Moreover, although ROS is induced upon trypanosome infection, indirect evidence suggests that susceptibility of tsetse to trypanosomes under diverse physiological conditions is not due to ROS (Table 7.1) although we cannot rule out that it may be involved with reactive nitrogen species to form peroxynitrite, a highly reactive anion. However, decreased H_2O_2 levels

in flies following blood feeding (due to haemoglobin degradation) suggests the involvement of ROS and antioxidant enzymes in tsetse midgut physiology following blood feeding.

Semi-quantitative RT-PCR was used to check for knockdown and transcript expression, and although attempts were made to use Western blots whenever suitable antibodies were found, quantitative qPCR would provide more robust results. Although transcript knockdown of Duox was successful, protein knockdown of Duox was unattainable when measured using the H_2O_2 assay. H_2O_2 assays including the use of a catalase inhibitor when dissecting should delay H_2O_2 from dissociating and should allow for a more accurate measurement of H_2O_2 after dsDuox has been injected into the flies. With regards to OXR1 knockdown, since the knockdown construct encompassed the most highly conserved and central region (TLDc domain), it is doubtful that different constructs would result in OXR1 knockdown. Perhaps feeding 9 µg or higher of dsRNA with the bloodmeal might be more effective at achieving knockdown with minimal mortality. Finally, in hindsight, the flies used in these experiments should have been standardized for age in order to make a more valuable comparison between experiments (Walshe *et al.* 2011).

The manual annotation of antioxidant genes onto the *Glossina* genome has enabled us to identify extended sequences, which can be confirmed by using rapid amplification of cDNA ends (RACE), amplifying the nucleic acid sequences from mRNA template at either the 3' or 5' end (Frohman *et al.* 1988). Successfully identifying alternative splicing of mRNA and validating exon-intron boundaries by using an exon-spanning PCR strategy can also be applied (Cerenius and Soderhall 2008).

Regrettably the *Glossina* genome was not available at the beginning of my degree program. If it had been available then, I would never have attempted to clone IRC since it seems that tsetse does not possess an IRC gene which is identifiable using obvious homologies. However, the release of the *Glossina* genome is an immense advancement for the insect community. It provides researchers with genomic tools central to further study tsetse flies. Although the release of the *Glossina* genome is very recent, collaborative efforts by geneticists, evolutionary and computational biologists will accelerate the pace of discovery.

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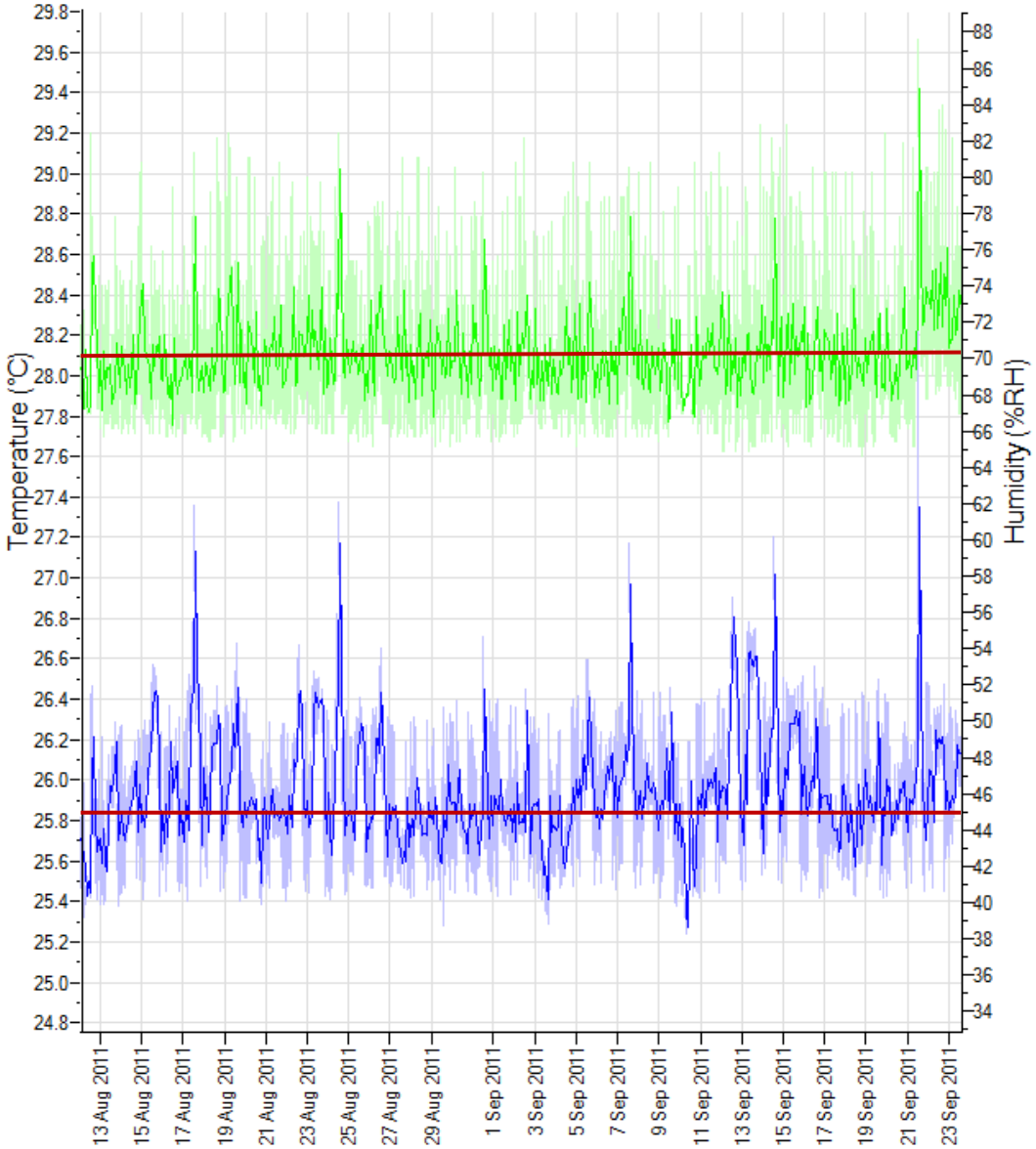
Appendix

Appendix I



Temperature and humidity of the insectary at LSTM were monitored as pupae were kept in this insectary. Humidity (%) (green) and temperature (blue) were monitored over a 2 months period with average humidity of 71% and average temperature of 25-28°C (red lines).

Appendix II

Extended nucleotide sequences of nitric oxide synthase (NOS), dual oxidase (Duox) and oxidation resistance 1 (OXR1). Highlighted in grey are the extended sequences and in white are the original sequences before extension.

Nucleotide sequence - NOS	Size
ATGTCACATTTCACTTCAATATTGGAAAACATACGTTTGGTGGCCATAAAACGTAGTCCTTTATCACAACAAAAACAATCAAACAAAGAGCAACAATCATCCACATCTCATCCACCAAGCAACACTGCTACGAT TACCATAACCGATACGGACCTGTTGCAGCAGCAACAAGCAGACATTCATAATGAATATAATAAAAGCAGTAAAGTGCTTAATACGACAACGTCTGCGAGAATAACGACATCAAAGACGATTTCGTCAAATGC ACATGCATATCAACACGAAGATCAAGCGGATAAAGGTGAAATCGCGAATAATAGTCACATTAGCGGAAATGTTGCAACTAATGCGATTAGCGGCACATCTGTTAAAGCACATTCGAAACGTCAGAGCGTTTC GGCACGTGGTTCAATTAGCTCCGGGGAAAATGTACGAACTTCACCGGGACGTGAACGTAACGTATCGAATCGTACCGCAAATCTTATTGCAGAAATTGATATCAATGGCAGTAATAGCAGCGTTTCCGGTGT AAAGAATTCCGTTACCAGAACACGTGATCTCTCACCTTCGCCCAAAAATCAACAACGTAGAATGTCAACAGATTTTCGAGCACGTGCCGGTAGCTTTATACATGTTGACGATGAAGGTCGCAGCATATTGATG CGCAAGCCAGTACGTTTAAAGAATATCGAAGGCAGGCCGGAAGTTTATGATACTTTGCATTTCAAGGGGTTTCAGATACTTTCTTGCTCGAAATCAACATGCATGAGCAGTGTTATGAATTTTGGTACAGCAC CAGTTGAAGCACGTAAATCAGAGGTGGTGTTAGAGCATGCCAAAGATTTTCTCGACCAATATTTTACGTCAATTAAAAGGTCTTCAAGTGCTGCTCACGAGGCACGTTGGAAGCAAGTGCGTCAAAGCATAG AAAGCACCGGGCATTATCAACTCACTGAAACTGAATTGATTTATGGCGCCAAATTAGCCTGGCGCAATTCCTCTCGTTGCATAGGTCGCATACAATGGTCCAAATTACAGGTATTTGATTGTCGGTATGTAACA ACTACCAGCGGAATGTTCGAGGCGATATGTAATCACATCAAATATGCTACCAATAAAGGAAATCTTAGGAGGTCAGCCATTACAATATTTCCGCAACGAACCGATGCACGTCACGATTATCGTATTTGGAATA CACAGCTTATCTCATATGCTGGCTATAAACAGAATGATGGAAAAATTATTGGAGATCCTTTGAATGTGGAATTTACGACAGTGGCATTAAAAATGAATTTGGACACACGTACACCAACCTCGTTATGGAAGGA CAAGGCAGTTGTCGAAGTTAATATTGCTGTACTTTATTCATATCAAAGCCGCAACGTCACCATCGTCGATCATCATACTGCTAGTGAAAGTTTTATGAAGCATTTCGAAAATGAATCAAAATTACGGAACGGCT GCCCGGCAGATTGGATTTGGATTGTGCCACCGTTGTCGGGTTCCATAACACCGGTATTCCATCAGGAGATGGCCTTGTACTATTTAAAACCATCATTTGAATATCAGGATCCGGCTTGGCGCACCCACATTTG GAAGAAAGGACGCGGTGATGGTAAAAGTAAAAAACCCAGGCGTAAATTTAATTTCAAACAAATTGCAAGGGCTGTGAAATTTACGTCGAAATTATTTGGACGTGCTTTATCAAAACGTATAAAAGCTACGGT ATTGTACGCCACCGAAACGGGCAAATCAGAAAAATATGCCAAGCAATTATGTGAACTACTAGGACATGCATTTAATGCGCAGATTTATTGCATGTCTGAATACGATATATCATCGATTGAACATGAAGCTCTC TTAATAGTTGTAGCGTCAACATTCGGTAATGGAGATCCTCCGGAAAACGGTGAATTGTTTTCTGTTTGTGTGTGTTTAATATTTTTTTTTAGTAATATTTGTGAAAATTTAATTGATAAAATTTCCTTTAAATGTT TTTTCAGCATTGGCGTCACATCTTCGAAATCCTTTATGAAAGCAAGCTCGAGGCAAGATTTGATTAAATTGCCTTTGCAACAGGTGAAGAAAATCGATAGATGGGATTCCTTAAGAGGCTCAACGTCAGACAC ATTTACAGAGGAGAACTTTGGACCATTATCTAATGTTAGGTTTGCAGTTTTTGCCTTGGGTTCTTCGGCCTATCCAAACTTCTGCGCCTTTGGCCAATACGTAGATAATATTTTAGGCGAACTAGGCGGAGAAC GGCTTCTGAAAGTTGCCTACGGCGATGAAATGTGCGGCCAAGAACAAACTTTTCGTAAATGGGCTCCAGAAGTATTTAAGGTGGCCTGCGAGACGTTTTGTTTAGATCCCGATGAAAATCTTTCGAATGCTTC GTTTGCTTTGCAGAATGAATCCTTATCTCTTAACACGGTACGTTTAGTGCCGTCTAAAGCGAAGGGTTCGTTAGAGCACTTGCTCTCGAAATATCATAACAAAAAAGTGACCTGTTGCCAAATCAAAAGAAATC CTCATAATCTTACTAATATGGATAATAGTGAAAAGACTACAATTCTTTTGGAAATACAAGCTGCCGGCCTGGACTATGAGCCCGGTGATCACGTTGGTGTATTTCCCGCAAATCGGCCAGAAATAGTTGACGG AATAATAAAACGTCTGGTTGGTGTCGAGGACCCCGATGAGGTCTTACAATTGCAATTGCTTAAGGAGAAACAAACATCGAATGGCATTTTCAAATGCTGGGAGTCACATGACAAAATACCTGCCGAGAGTTT AAGGACTTTGCTTACCCGTTTTTTTGATATAACAACACCTCCGTCAAGGCAATTCTTAACGTTGTTAAGTGGATTTTGTGAAGATAATCATGACGTTGAACGTTTGCAATTACTGATCAATGATTCGTCGGCTTA TGAAGACTGGCGGCACTGGCGATTGCCACACCTGCTAGAAGTATTGGAAGAATTTCCGTCATGTAAACCACCGGCTTCCTTAGTATTGGCCAATTTAATGTCTTTACAATCAAGATTCTATTCGATATCCTCTTC GCCGAGCAAGGTAAACAACGAAATCCATTTGACGGTGGCCGTTGTTAAGTATCGAAGCGAGGATGGAAATGGTGATGAACGTTACGGTGTCTGTTCCAATTACTTGGCTGCTCTCAAGGCTCAGGATGATTT ATTTATATTTGTACGTAGTGCTCCCGGCTTTCATTTGCCTTCTGATCCTCTCAATCCCATTGTGCTAATTGGTCCCGGTACCGGTGTTGCACCATTCCGATCTTTCTGGCAAGAATTTGAAATGAAACGCGAAAT GCAAGCAAACTGCCAGCTACCAAAAGTATGGCTTTTCTTTGGTTGTCGTAATAGCAAAGTCAATCTGTATGCCGATGAGAAAGAACGCCTAATCAAAGAGAATATATTAAATCGTATCTTTTTAGCTTTGTCCA GAGAA	3,471 bp

Nucleotide sequence - Duox	Size
AGCCACTTGGTCCGGAAAGCACCGCCGTCATACTCTGATGGTGTTTATGCTTTGGCGGGTTTAAATCGCCCTTCTACTAGACGACTAAGCCGTTTGTTTATGAGAGGACGAGATGGCCTAGGATCAAAGTAT AATCGAACCGCATTATTGGCATTTTTTGGTCAGGTTGTGGCCAACGAAATTGTGATGGCATCTGAGTCGGGATGTCCCATTGAAATGCATCGCATTGAAATCGAAAAGTGTGATGAAATGTACGATAAAGCC TGTAGGGGAGACAAATATATACCTTTCCATCGCGCTGCCTATGACCGGAATACAGGTTCGAGTCCTAACGCTCCAAGAGAACAAATTAATCAAATGTCAGCCTGGATTGACGGTAGCTTTATATACAGCACTT CGGAGGCATGGCTAAATGCAATGCGTTCATTTCATAACGGTTCTCTACTGACAGATAAAAGTGGAAAATTGCCCATACGAAATACCATGAGAGTTCCGTTATTCAATAACCCGGTACCGAACGTTATGAAAA TGTTGAGTCCTGAGAGATTATTTGCAGTACTTGGTGATCCACGAACCAATCAAAATCCAGCAGTACTTTCATTTGCTATATTGTTCCTGCGTTGGCACAACACGTGGGCTCAGCGCGTTAAACAAGCGCATCC TGGTTGGAGTGATGAGGATATATTTCAGAAGGCCCGTCGTTATGTTATTGCGAGTTTGCAGAATGTTATCATGTACGAGTACTTACCAGCTTTTTTGGCTTCTGAGATACCGCCTTATGAAGGTTACAAACAA GATATTCATCCTGGTGTGGGACATATTTTTCAGGCTGCAGCCTTCCGATTTGGTCATACTATGATACCGCCAGGTATATATCGCCGAGACGCTAATTGTAATTATAAGGCCACAGCGATGGGTTATCCTGCCA TAAGGCTTTGTTCCACTTGGTGGGATTCAAGCGACTTTCTAGCAGACACCAAAGTCGAAGAAATTCTCATGGGCTTATCTTCGCAAATATCAGAACGCGAAGATCCTGTTCTTTGCTCCGATGTGAGAGATAA ACTATTTGGTCCTATGGAATTTACGCGTAGAGATTTAGGTGCTCTAAATATAATGCGTGGTCGAGATAATGGTTTGCCTGATTATAATAGCGCTCGCGTAGCTTTTGGCTTACTTGAGCACAATAATTGGACT GACATCAATCCTGACCTATTTTCGAAGCAACCGGAATTGCTGGAGAGTCTTATATCAGCCTACAACAATCGATTGGATGACGTTGATGTGTATGTGGGTGGAATGCTTGAGTCCTATGGTCAACCGGGTGAG CTATTTAGTACGGTTATCAAAGAGCAATTTCAGAGACTCAGAGATGCCGACCGTTTCTGGTTTGAGAATGATAAAAACGGTATATTTGCACCTGAAGAAATCGAAGAGATCCGTAAAATACGCATGTATGAC ATTATTGTAAACAGCACTGATATTAATCCGCAGCATATTCAAAAAAATGTTTTCATATGGCATAAGGGTGATCCATGTCCGCAACCAATGCAATTAAACGCTACTGAATTGGAGCCATGCACTCACTTAGAAG GTTACGATTATTTTTCGGGGTCTGAACTTATGTTTATTTATGTTTGCGTTTTTCTGGGATTTGTACCAATTCTATGTGCTGGAGCTAGTTATTGCGTTGTCAAATTGCAAAATAGTAAGCGTCGCAAATTGAAGA TACGTCAGGAGGCCTTGCGATCTCCTCAACACAAGGGTTCTGTAGACAAAATGTTAGCTCGCGAATGGTTACACGCAAATCACAAACGCCTTGTCACTGTTAAATTTGGACCGGAAGCTGCCATTTACACAG TAGACCGTAAGGGCGAAAAACTGAGAACTTTTAGTCTTAAAAATATCGATTTGGTTACAGTAGAGGAATCAGCTACAAATCATATTAAAAAGAAACCATACATCTTGTTGCGAGTGCCCAATGACCACGATT TGGTTCTAGAGCTTGAGTCCTATGGAGCTCGTAGAAAATTCGTCAAAAAACTGGAGGATTTTCTGATATTGCATAAAAAAGAAATGACTTTGGTCGAAGTAAATCGTGACATAATGCTAGCTAGAGCCGAAA CTCGCGAAAGAAGGCAAAAACGACTGGAATATTTTTTTCGCGAAGCTTACGCCTTAACATTCGGTTTACGACCCGGAGAAAGACGACGTCGTTCAGACGCTTCCACAGATGGTGAGGTAATGACAGTAATG CGCACCAGCTTGTCAAAAGCTGAATTTGCCGCTGCTCTCGGCATGAAACCGAACGACATGTTCGTTCGTAAAATGTTTAATATTGTCGATAAAGATCAAGATAGTCGAATTAGTTTTCAAGAGTTTTTAGAAA CGGTCGTTTTATTTTCACGAGGCAAAACAGACGATAAACTACGTATTATTTTCGATATGTGCGATAATGATCGCAACGGAGTCATTGACAAAGGTGAGCTTAGTGAAATGATGCGTTCTCTAGTTGAAATAG CTCGTACTACAAGCCTCGGTGATGATCAAGTAACTGAACTGATTGATGGAATGTTTCAAGATGTCGGCTTGGAGCACAAAAATCATTTAACCTATCAGGATTTTAAACTGATGATGAAAGAATACAAGGGTG ATTTTGTAGCTATTGGCTTGGACTGCAAAGGGGCTAAGCAGAATTTTCTGGATACCTCGACAAATGTAGCCAGGATGACTTCATTCAATATTGAGCCAATGCAGGAAAGGCCCCGTTATTGGATGGAAGTGA AATGGGATAGTTACATAACATTTCTAGAAGAAAACCGTCAAAATATTTTCTATTTGTTCTTATTTTATGTTATAACAATTGTTTTGTTCGTTGAACGATTTATAGATTATTCATTCATGGCAGAACATACAGATTT GCGCCATATTATGGGTGTTGGCATTGCTATCACTCGAGGTTCAGCCGCCTCATTGTCATTTTGTTATTCCTTGCTGTTGCTTACGATGTCCAGGAATCTAATAACAAAACTAAAGGAATTTCCTATACAGCAGT ATATTCCTTTAGATTCGCACATACAGTTTCACAAAATTGCTGCCTGCACGGCTCTATTTTTTTCTGTTCTACACACTGTGGGCCATATTGTTAACTTTTATCATGTGTCCACGCAATCGCATGAGAACTTGAGAT GTCTAACTCGTGAAGTGCACTTTGCATCAGACTATAAACCGGATATAACATTTTGGCTCTTTCAAACAGTGACAGGTGTCCTGCTGTTTATTATTATGTGCATCATATTCGTTTTCGCTCACCCTACCATTCGCA AAAAGGCGTACACTTTTTTTTGGAATATGCATTCACTTTACATTGCTCTGTATTTGCTAAGTCTCATACATGGGTTAGCTCGGCTCACAGGTCCTCCGCGTTTTTGGATGTTTTTTCTTGGACCCGGTGTAATAT ACACGCTGGATAAAATTGTATCACTACGTACCAAGTATATGGCTCTGGATGTAATTGAAACCGAGTTGCTGCCATCCGACGTTATCAAAATAAAATTCTATCGTCCACCTAACCTAAAGTATCTTTCTGGCCAG TGGGTTCGACTGTCATGTACTGCGTTTCAACCTCACGAAATGCATAGTTTTACG	3,645 bp

Nucleotide sequence – OXR1	Size
AAGTCGTCTCTACCGACTATACCTACTATAAATTACACGGTAGGCAATCGGGACACTTTAACTTCGGTAGCTGCTCGATTCGATACCACTCCATCTGAATTAACACATTTAAATCGTTTGAATAGTTCGTTTATT TATCCGGGCCAACAATTACTGGTACCCGATAAGAGTGCTAAAGATGACATATTGGAGAGTCTGCGTCCTGGTTCTCCAAAGCCTGGCCGCATAGAAAGGATTGATGTTAATGACCGCAGTGATATGAATAA GACGGATGATCCTATTATTACTCAGCGCTTTCTCAAAATCAATGTACGTCATATAACTGATGGTCAGGGAGTCGTGGGCGGTGTTCTTTTAGTTACGCCGAACGCGGTAATGTTTGATCCTAATGTCTCGGAT CCCCTGGTTATTGAACATGGTCCAGAGAGCTACGGCGTAATCGCTCCCATGGAATTGTGCGTTAATGCGGCCATATTTCATGATATTGCCCATATGCGCGTATCTGGTGGAGCAGTTATTAGTGCAGATAACA CAAACGCAACTGAAAAACCAGAAATTTATTATCCAAAAGCAGTATTAGAAGCTACGCTAGAGGAGAGACGTAAAAGCCTTCTCGATCATCATTGGGCTATACCAAGTAAAGACAGGGATTTGGAACAACTA GAACAGCTAACGAAACAATCGTGTTACGATTCTGGCATCGATATACGCGAGCCGATACCAAATGTACAACCGATACCAAAGAAAACCGTTTACAGTGATGCTGATATTGTATTGAGCTCTGATTGGGTGCCA CCGAAACCTATATCAACCACACCACTTAGTGAATCGCCCCCGCGTTCTAGTGGTATGGCGACTTGTCAAAGCCTAGATGCAGGCACCGGTGGAACACGTAAAAAGACCTCAAGCGTCAGTTTCAGCGTAGAC GATATGTTGAAACGCCTGTCGTATCCTTTAACTTGGGTCGAAGGTTTGACCGGTGATGCAACCTCTAGTGCGGTTGCTGGTGTTAGCAAATCAATAGACACTGATTCAGCTCCTAACACCGGGGATTCGAATC AGAGTGTGTTTTCAAAGGTGTTTTCAAGGCGTTCCTCAATTGGTACCTTCATGCGTCCCACATCTTCGGAAGGTAGTTCGTCAGCAATTAAAACAAAAGAAGTTAAAGTTCAACCGAAATTAGATTATCGTTC AATGGTTTCTGTGGACGACAAGCCAGAATTGTTTGTTAGTGTTGACAGTAAGCTTATACCTCGACCAGCTCGTGCTTGTCCTGATCCACCGTTGTACCTACGCTTACGTATGGGTAAGCCGACCGGCAAAACC ATACCATTACCCACCTCGGTCATGTCGTATGGCAAAAATAAATTGAGACCTGAATACTGGTTTAGTGTACCTAAAAATCGTGTTGATGAATTATATCGTTTTATGAATACCTGGGTGCCTCATCTATATGGTGA ATTAGATGAAGAACAAATCAAGAATCGTGGTTTTGAATTAATACAAGATGATACGGAATGGACACAGAGTGGTACCAAAAAATCAGGTTCACGCTCTAATAGCCAAGAGGGCGAAGAGATTAGCGATTTAA CACGCGAATCATGGGAGGTGTTGTCTATGAGCAATGATGACTACCGCAAAGCGTCCATATTCCAAACGGGATCGTTCGATTTAGACTTTCCGATACCTGAGCTAATTGGTACCACCGAAATTTTAACCGAAG AGCATCGCGAAAAACTCTGCAGTCATTTACCTGCTCGAGCAGAAGGCTATTCCTGGTCTTTGGTATTTAGTACATCTTTACATGGATTCTCGTTAAATTCATTATATCGTAAAATGCAACGTCTCGAGAGTCCC ATACTGATTGTTATTGAAGATACAGATCACAATGTTTTTGGCGCTTTAACTTCTTGTTCCCTGCACGTATCGGATCACTTTTATGGTAACGGTGAATCGTTGTTATATAAATTTAATCCCAGCTTTAAAGTATTC CATTGGACTGGGGAAAATCTTTATTTTATTAAAGGCAATGTTGAAAGTCTCTTAGTTGGTGCCGGAAACGGCCGATTCGGTTTATGGTTAGATGGTGATTTAAATCAGGGTCGCTCGCAATCGTGCAGCACG TACGGCAATGAGCCTTTGGCGCCACAAGAAGACTTTGTTATTAAGACTCTTGAAGGCTGGGCATTTGTTTAA	2,196 bp